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Resumen por la autora, Mary J. Hogue

Una comparación entre una amiba, Vahlkampfia patuxent, y las células de un cultivo de tejidos.

Los fibroblastos y células blancas de la sangre de embriones de pollo fueron cultivados en un medio de cultivo de tejidos y comparados con la amiba Vahlkampfia patuxent. Las amibas fueron introducidas en los cultivos de tejidos, estudiándose las reacciones de ambas clases de células bajo la acción de los colorantes vitales. Las amibas son cuatro veces más sensitivas que las células del cultivo. Se compararon las mitocondrias de las células. Los granos de melanina de la retina del embrión fueron ingeridos por las amibas y las células del tejido cultivado. La autora describe el método de ingestión de dichas partículas por la amiba. Son expelidas sin digerir. Las células del tejido murieron sin haber expelido los gránulos de pigmento. La motilidad se considera como un criterior de la vida de la amiba durante el estado de trofozoito, mientras que en el caso de la célula cultivada su reacción hacia los colorantes vitales se ha usado para comprobar si está todavía viva ossi ha muerto.

Translation by José F. Nonides
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AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
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A COMPARISON OF AN AMOEBA, VAHLKAMPFIA PATUXENT, WITH TISSUE-CULTURE CELLS

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THREE FIGURES

INTRODUCTION

While working with tissue cells grown in culture medium I was much impressed with the similarity of their appearance to that of amoebae grown on agar-agar plates, and determined to make a comparative study of these two kinds of cells. The particular cells used for this study were the fibroblasts and the white blood cells from the embryonic chick and an amoeba, Vahlkampfia patuxent (Hogue, '21), which is a salt-water form, parasitic in the digestive tract of the oyster.

The tissue cells were grown at 39° in hanging drops of Locke-Lewis solution (Lewis and Lewis, '15) inverted over hard vaselin rings on depression slides.[1] The amoebae were taken from agar-agar plates made up with 0.7 per cent sodium chloride solution, with 0.4 per cent peptone, and kept at room temperature.

The comparison of these two kinds of cells was made by studying permanently stained preparations, by introducing the amoebae into the cultures where the tissue cells were growing and here studying their different reactions to various vital stains and pigment granules and by further testing out the effect of these stains and pigment granules on the different kinds of cells in their own culture media.

[1] Mrs. Lewis kindly furnished me with tissue cultures of connective tissue and spleen.

MORPHOLOGY AND MOVEMENT

At first the most noticeable difference between these cells is their diversity in size. The amoebae averaged 21 μ in length and were much smaller than the fibroblasts which measured 50 μ in length. The exact size of the fibroblasts is hard to determine, as their processes are so thin and fine that their farthest limits are difficult to determine. The amoebae always have a definite outline, though they are continually changing shape. Still another difference is that the amoebae can be observed to move rapidly, while the movement of the fibroblasts is too slow to be noticeable, though they do move as can be seen by watching the growth of the culture from day to day. The amoeba moves by lunging in one direction and then in another. The cytoplasm is thicker and denser than in the fibroblasts where the processes are very thin and flat against the cover-slip.

In order to study the two kinds of cells together, the amoebae and the bacteria growing with them were introduced by means of a platinum loop on the cover-slip where the tissue cells had been growing for from twenty-four to forty-eight hours. The amoebae began at once to crawl actively around and over the fibroblasts without apparently disturbing them.

The tissue cells had to be kept at 39°C. The amoebae ordinarily live at room temperature, though, as I have already shown (Hogue, '21), they can be kept at 35°C. for twenty-four hours, after which they showed a decided tendency to encyst some time during the following week, the encystment not following immediately on their removal from the high temperature. When the amoebae were brought into the warm box, where the cultures were examined microscopically, their rate of motion was greatly increased. They were now moving rapidly, and continued to do so as long as they were kept at this high temperature.

REACTION TO BACTERIA

The presence of the bacteria, which were chiefly non-pathogenic bacilli serving as food for the amoebae, was very harmful to the tissue cells. They evidently could not stand the toxic effect of the bacterial waste products. In a few instances some

of the fibroblasts lived twenty-four hours after the introduction of the amoebae and the bacteria into the tissue culture, though most of the fibroblasts pulled in their processes and contracted into small oblong masses within a few hours after the introduction of the amoebae and bacteria. Later they went to pieces, while the amoebae moved about quite normally in this new medium (Locke-Lewis), and lived for over two weeks, when the experiment was discontinued.

REACTIONS TO STAINS

Neutral red

In trying out the effect of vital stains on the cells it was found that a very weak solution of neutral red was sufficient to give an excellent stain. When neutral red 1 to 10,000, the proportion used for tissue-culture cells, was used with the amoebae, they would immediately round up, stain red, and die. This solution was sometimes allowed to dry on a cover-slip, which was then put down on the drop of Locke or normal salt solution containing the amoebae. The neutral red would gradually diffuse into the medium and stain the neutral-red granules and vacuoles of the amoebae without immediately killing them. Experiments with neutral red of different strengths were made; 1 to 40,000, 1 to 60,000, and 1 to 80,000 in Locke solution were all good and not sufficiently strong to kill the animals. Amoebae were kept in hanging drops of Locke solution with neutral red 1 to 40,000 for nine days; at the end of this time they were moving slowly and had a few neutral-red granules. In another set of experiments the amoebae were kept in Locke solution with neutral red 1 to 80,000 for twenty-four hours. At the end of this time most of them had lost the stain, so Locke with neutral red 1 to 60,000 was added, and they continued active with stained neutral-red granules for seven more days, when the experiment was discontinued. This is especially interesting, as in tissue cultures the disappearance of the color from the neutral-red granules was always taken as an indication that the cells were dead. With the amoeba after the color has disappeared the granules can be restained with neutral red and the amoeba is still normally active.

The disappearance of the neutral red from the amoeba seems to be due to oxidation. One small amoeba was watched for five hours. At 9:00 in the morning it contained fifteen neutral-red granules. The color gradually faded from these granules until by 11:30 only one granule had the neutral-red color and by 12:00 this color had disappeared. The granules themselves did not dissolve, but could be still seen in the amoebae. Unfortunately, janus black no. 2 had been also used, and this eventually kills the cells. However, at 4:30, this particular amoeba was still

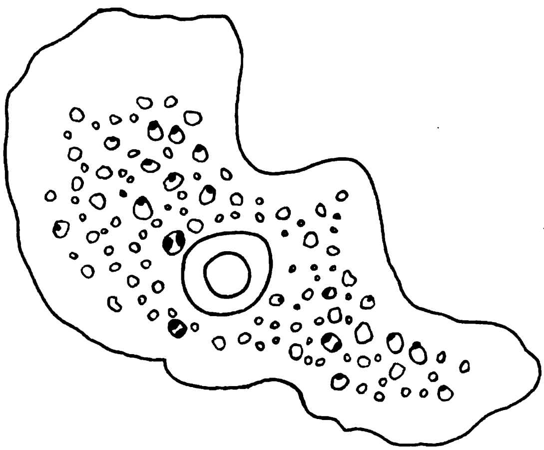


Fig. 1 Amoeba showing neutral-red granules of different sizes and neutral-red vacuoles containing one or two neutral-red granules. 12.5 ocular, 1.9 oil immersion.

very active with numerous stained mitochondria, but the next morning it was dead.

The neutral-red granules and vacuoles were of varying size and number. Some amoebae had many small granules and many large vacuoles, which contained from one to two granules (fig. 1). In the fibroblasts one frequently finds neutral-red channels, but they have never been observed in the amoebae.

Brilliant cresyl blue 2 b

The amoebae were much more sensitive to this stain than were the tissue-culture cells. One drop of a weak solution was added

to the tissue culture. The fibroblasts responded at once in the usual way (W. H. Lewis, '19) to this stain. Many of the vacuoles stained pink with one or more purple granules. The vacuoles were grouped around the nucleus and centriole, just like the neutral-red granules. This same solution applied to the amoebae stained the cytoplasm a pale blue, the granules a purple blue, and the karyosome of the nucleus a purple blue with a clear area in the center. The nucleus with its karyosome and clear area were pulled away from the endoplasm out into the ectoplasm. The amoeba moved slowly and sent out many large clear pseudopodia, a kind of lunging movement similar to blebs in tissue cells. Within half an hour the granular endoplasm had contracted into a solid blue mass of irregular shape, the nucleus remaining in the ectoplasm. Motion had stopped and the amoeba was dead.

This solution of brilliant cresyl blue was diluted one-half with Locke solution and applied to the amoebae. This time about half of the endoplasmic granules took the deep blue stain, the other half remained unstained and were quite refractile. The karyosome stained purple as before, but with a bluish purple area around it and the cytoplasm was a pale blue. The amoebae moved actively, but in a short time the granules lost their color and the nucleus and cytoplasm stained more deeply and the amoebae were dead.

When the solution was again diluted one-half and added to a new hanging drop of amoebae, the effect was the same as that on the tissue cells with a solution four times its strength. The nucleus was not stained. The endoplasm contained purple granules as well as the pink vacuoles with their purple granules. The amoebae moved rapidly and progressed normally.

Methylene blue

The solution of methylene blue, when added to the tissue cells, did not stain the nucleus nor cytoplasm. The vacuoles stained blue and the one or more granules within them took a deep blue stain. Blebs were forming on many of the cells. The same solution added to the amoebae stained the karyosome a deep

blue, the granules a deep blue, and the cytoplasm blue. The nuclear area around the karyosome remained clear and the amoebae moved freely. Later they rounded up into blue balls surrounded by a lighter blue ectoplasmic area. In this area the nucleus was usually found away from the endoplasm. The amoebae were dead.

When the solution of methylene blue was diluted one-half, the effect on the amoebae was the same, though the animals did not die so quickly. When it was diluted one-fourth of the original strength, it stained the amoebae the same as the original solution had stained the tissue-culture cells. The cytoplasm was clear, the nucleus unstained and invisible except as a clear area in the endoplasm free from granules. Many of the granules were colored blue, though not all took the stain. To this stain also the amoeba cell is four times as sensitive as the tissue-culture cell.

MITOCHONDRIA

The amoebae were treated with janus black no. 2. A very weak solution was used, as otherwise the amoebae would contract and die without showing the mitochondria at all plainly, though the nucleus stained well.

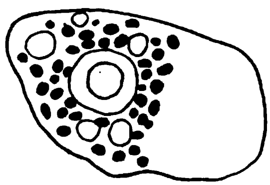
When the amoebae stained slowly the mitochondria appeared as small short rods, like bacilli, in among the neutral-red granules (fig. 2). Some of the mitochondria were round, and frequently after they had been stained for some time groups of three or four small mitochondria would be found in among the larger ones (fig. 3). No long branching mitochondria were found in the amoebae, though they are very common in the fibroblasts. The mitochondria retained the stain and were visible as long as the amoebae were alive. This stain seemed to have a stupefying effect, as the amoebae died with their pseudopodia extended instead of rolling up into balls.

The mitochondria, when the amoeba is dying, have very marked brownian movement. In the later stages of death the mitochondria move out into the clear ectoplasm which before has been free from granules of all kinds. This does not occur in the tissue-culture cells, as I have already shown (Hogue, '19).

REACTION TO MELANIN PIGMENT GRANULES

In studying the amoeba's reaction to melanin pigment granules, I first introduced the amoebae into tissue cultures of the spleen which had been growing with the melanin pigment from the retina of a chick embryo for from twenty-four to forty-eight hours. Later I simply put the granules into hanging drops of 0.7 per cent normal salt solution with the amoebae.

As soon as the amoebae recovered from being transferred and were moving about with their large ectoplasmic pseudopodia, they began to take in the pigment granules. The amoebae reacted differently to these granules which were in great numbers



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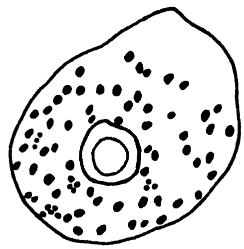

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Fig. 2 Amoeba stained with neutral red and janus black no. 2. The neutral-red granules are clear, the mitochondria are solid black. 12.5 ocular, 1.9 oil immersion.

Fig. 3 Amoeba stained with janus black no. 2. The mitochondria are oblong and a few small ones are arranged in groups. 12.5 ocular, 1.9 oil immersion.

and were showing brownian movement. Some of the amoebae at once took in large numbers of the granules, others only a few, and still others moved about freely among the granules without taking in any of them. There was evidently some difference in the physiological condition of the amoebae at that time.

Many of the granules were oblong, so that it was easy to determine their movement in the cells. They always entered by way of the anterior pseudopodium, whether it was one large pseudopodium or two smaller ones. The amoeba took in the granules in the same manner that it takes in its food. The granules had to be in the same plane as the advancing pseudopodium. If they were above it or below it the amoeba simply went past it

without taking it in. The granules could lie in any position in relation to the advancing pseudopodium. They were horizontal and perpendicular to it and at times formed an angle with it. Often they were swung around from a perpendicular to a horizontal position and vice versa, and sometimes they swung from a perpendicular or horizontal position to a slanting one and vice versa as they entered the ectoplasm. Once in the ectoplasm their position was constant until they reached the endoplasm. As soon as they were there, they were whirled about by the endoplasmic currents.

As a rule, only one granule was taken in at a time, though I have seen a group of three go in at once, and frequently two granules will be taken in at different parts of the pseudopodium at the same time. The amoeba is capable of taking in a large number of these granules. Four hours after the pigment granules were introduced into the hanging drop of amoebae, the amoebae were filled with the granules. They had at least a hundred granules and resembled the pigment cells of the retina, except for the fact that they were moving rapidly.

The amoeba takes the granules in by simply pushing against the granules with such force that the granule pierces the ectoplasm. For this reason the granule to be taken in must be in the path of the advancing amoeba. If it is to one side of the anterior pseudopodium it is passed by; if it is near the side of the pseudopodium, but still anterior to it, it may be taken in or it may be pushed off farther to the side, depending on how far to the side it is and also on how rapidly the amoeba is moving. If it is moving very fast the granules near the outer edge of the anterior pseudopodium are much more likely to be pushed away than to be taken in, or they may remain attached to the amoeba like the spines of a sea-urchin and be passed back along the edge until they reach the posterior end. Here they frequently remain attached for a long time, but are never taken in at this point. When the amoebae have been in a medium full of these melanin granules for some time many of them are found carrying at least fifty or more granules on their pointed posterior ends. They fairly bristle with the pigment granules at this point.

After the granules have entered the endoplasm, they circulate freely through it. Sometimes two or more will unite and occasionally a vacuole will form about these fused granules. Sometimes there will be as many as three or four clumps of granules in one vacuole. These vacuoles are heavy and lag behind in the posterior part of the amoeba. Eventually they come in contact with the outer edge of the amoeba. After that they soon break, discharging the pigment granules to the outside. Some of the granules remain attached to the amoeba, others, more forcibly ejected, float away in the medium. In this way the amoeba gets rid of the melanin pigment. After twenty-four hours most of the amoebae had given off the granules and were moving about freely among the granules without taking them in again, at least not in such large quantities as when the granules were first introduced into the hanging drop. The amoebae never digested the melanin pigment.

Small mononuclear blood cells were present in large numbers in the tissue cultures of spleen. These the amoebae took in, carried them about for a while, and then gave them off. Several of them would often remain sticking to the posterior end of the amoeba along with many pigment granules. All this weight did not seem to retard the movement of the amoeba.

These results are somewhat similar to those obtained by Smith ('21) with tissue-culture cells, except that the tissue cells take in the melanin pigment at all parts and the pigment granule must always be horizontal to the surface of the cell where it is entering. He notes the formation of vacuoles, but could not determine the ultimate fate of the granules, as the life of the tissue-culture cells is short, and they died without discharging or digesting the granules.

DEATH

Ordinarily when the nucleus and cytoplasm of a tissue-culture cell have taken methylene-blue or brilliant cresyl-blue or neutral-red stain, the cell is said to be dead. Again in tissue-culture cells, when the color of the neutral-red granules fades and they become clear, the cell is thought to be dead (Lewis, '19). With

the amoeba a different standard must be used. In one experiment already cited the amoebae were treated with neutral red and with janus black no. 2 at 9:30 in the morning. The neutral red color had disappeared at 12:00, noon, but at 4:30 in the afternoon the amoebae were still moving actively. The next day they were dead.

This is also true of both brilliant cresyl blue and methylene blue. The amoeba moves rapidly after its nucleus and cytoplasm have both become stained. It is evident, therefore, that these old criteria of the death of a cell cannot be applied to an amoeba which still continues to move. Indeed, one can wonder whether the tissue-culture cells are really dead when their nuclei and cytoplasm have taken these stains.

When these amoebae are alive they are actively sending out pseudopodia. Usually these come from only one part of the cell at a time, though any part may send them out. The amoebae not only send out pseudopodia, but they move rapidly. As the animals begin to die, progression is less rapid, though the pseudopodia are formed very quickly. Then gradually the pseudopodia are formed more slowly, then only at one end of the amoeba, and eventually they are simply blebs which flow from one side of the amoeba to the other until finally all motion stops and the amoeba is dead.

From the fact that locomotion and motion become gradually less and less as the amoeba is dying, it seems evident that the motion of an amoeba and not its reaction to vital stains should be the ultimate criterion of life in the trophozoite stage.

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Resumen por el autor, Theophilus S. Painter

Estudios sobre la espermatogénesis de los Mamíferos.

I. La espermatogénesis del opossum (Didelphys virginiana)

El presente trabajo ha sido emprendido con el fín de aclarar los resultados contradictorios sobre el número de cromosomas obtenidos por Jordan y Hartman. El autor ha hallado 22 cromosomas en las espermatogonias. Los dos más pequeños constituyen un complejo cromosómico X-Y típico, que puede observarse durante la maduración. En la primera división madurativa existen en el huso 11 cromosomas, diez de los cuales son tetradas y el otro el cromosoma X-Y. Los elementos X e Y se separan durante esta división de tal modo que los espermatocitos secundarios llevan 10 autosomas más X o 10 autosomas más Y. Ambos tipos de cromosomas sexuales se dividen ecuacionalmente en la segunda división de maduración.

Las células somáticas de embriones, en vías de división, presentan en todo caso 22 cromosomas, pero en el caso de los machos el complejo X-Y existe y en las hembras encontramos 2X. El autor llega a la conclusión que Jordan no pudo hallar más de 17 cromosomas espermatogoniales a consecuencia de la fusión de los cromosomas en su material. El "cromosoma accesorio" descrito por dicho autor es probablemente una tetrada desplazada. El error de Hartman sobre el número de cromosomas se debe a una división longitudinal de una de las tetradas en el huso del segundo glóbulo polar.

Translation by José F. Nonides
Cornell Medical College, New York

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STUDIES IN MAMMALIAN SPERMATOGENESIS

I. THE SPERMATOGENESIS OF THE OPOSSUM (DIDELPHYS VIRGINIANA)[1]

THEOPHILUS S. PAINTER

EIGHT TEXT FIGURES AND THREE PLATES (TWENTY FIGURES)

INTRODUCTION

The opossum, along with many other mammals, is a form for which the diploid or somatic chromosome number is a matter in dispute. In 1911 Jordan gave an account of the spermatogenesis of this animal. His results as they touch the chromosomes are briefly as follows: 17 chromosomes were found in dividing spermatogonia; in the primary spermatocytes 9 chromosomes were present—one of these was bipartite in form and passed apparently undivided to one pole of the spindle; in part of the second spermatocytes this bipartite element was found, in others it was lacking. Because of its behavior and distribution, Jordon interpreted this bipartite chromosome as the true sex-chromosome, and from his description it is clear that one half of the sperm carry this element and one half lack it; in other words, that the male opossum possessed the X–O type of sex-chromosome. No further work upon the male opossum has appeared except a short note by the author (Science, May 27, 1921) in which the main results of this paper were outlined.

Recently, Hartman ('19) working upon the early stages of opossum embryos, reports that in the ova (polar-body spindles) he finds from 10 to 12 chromosomes. Since 12 chromosomes are found in several spindles where the chromosomes are most favorably placed for counting, Hartman concludes that this is the true reduced or haploid number. From this it would follow of

[1] Contribution no. 150, from the Department of Zoölogy, University of Texas, Austin, Texas.

course that the diploid or somatic chromosome number for the female opossum is 24.[2]

The discrepancy between Jordan's and Hartman's counts amounted to 6 chromosomes—a number far too large to be explained on the basis of differences in the sex-chromosome complex.

The author undertook the present work with a view of determining, if possible, the cause for the difference in the chromosome counts of the two investigators whose work has just been cited. The problem, however, is not simply a matter of chromosome numbers, but one which has a broad and very important bearing upon the whole subject of spermatogenesis and sex-determination in the mammals. For the discrepancy in the works upon the opossum is typical of the confusion in the literature over the number of chromosomes for many other mammals; in fact, there is less difference in the counts for the opossum (6 chromosomes) than for such forms as the pig (where 18 and 40 have been given as the diploid numbers), or for the male of the human species, which has been variously reported to have 22, 24, and 47 chromosomes.[3] It is clear that, as long as the total number of chromosomes possessed by an animal is a matter of doubt, we cannot safely accept any conclusions regarding the sex-chromosomes of that form.

In a paper on the spermatogenesis of lizards, the author ('21a) has shown that the presence of a bipartite body at one end of a spindle in maturation divisions does not necessarily mean that it is a sex-chromosome as has been so often assumed in vertebrate spermatogenesis. It is frequently either a whole tetrad or half of a tetrad which has divided, the other half remaining in the equatorial plane of the spindle. In the present paper (p. 30) a number of such false sex-chromosomes

[2] Hill ('17), working on the South American opossum, Didelphys aurita, estimated that there were 12 (haploid) chromosomes in the egg of this species.

[3] Wodsedalek ('13), gives 18 as the diploid chromosome number for the pig, while Hance ('17) shows that there are 40 chromosomes in both the germinal and somatic cells. Among the recent counts for the male of Homo sapiens we have the following: Guyer ('10), 22; Montgomery ('12), 23 or 24; von Winiwarter ('12), 47; Wieman ('17), 24.

are shown (text fig. 7). It is not to be doubted but that some of the 'accessory chromosomes' reported by different investigators of mammalian spermatogenesis are of this false type.

In addition to the question of chromosome number and of sex-determination in the opossum, a number of points of rather special interest to cytologists are touched upon; chief among these is the phenomena of so-called 'double reduction' first described by Guyer ('09) and reported for the opossum by Jordan.

MATERIAL AND METHODS

The present study is based upon the testes of four opossums and upon the dividing somatic cells of eight embryos.

In Texas the breeding season for the opossum begins in January. The males, upon which this study was made, were operated on as follows: one male on October 8, 1920; one male on November 15, 1920, and two males on January 14, 1921. Mature sperm, dividing spermatogonia, and cells in all stages of maturation were found in all the males but the maturation stages were more abundant in the individuals whose testes were preserved in January.

In the first male, an attempt was made to secure fixation by injecting the fixing fluid into the blood system, as Allen ('19) recommends. Only partial success attended the use of this method. Spermatogonial chromosomes (fig. 1) show up with sharpness, but the first and second spermatocyte divisions are badly masked by chromosome fusion. This may be due to the ether used in anaesthetizing the animal. In the other cases, no ether was used. The males were tied down, the scrotal sacs cut open with a razor and the testis removed. The testis was cut into several pieces with scissors, and then one or more of these pieces dropped into the fixing fluid, and the tubules quickly teased apart, so that complete penetration would be secured. A period of less than thirty seconds elapsed between the time the tubules were receiving blood from the animal and were being bathed in the fixing fluid.

Two preserving fluids were used. One was modified Bouin's solution, suggested by Allen ('16, '19), warmed to about 38°C.

The other was the cold Flemming method, with urea, as recommended by Hance ('17). The modified Bouin's fluid gave superb preparations for all stages of maturation, and was the more satisfactory of the two preservatives. However, it is my experience that for spermatogonial and somatic divisions, no preservative can surpass the results obtained by cold Flemming to which urea is added.[4]

A great deal of care was used in handling the material after preservation. When modified Bouin was used, the suggestions of Allen ('19) for washing and dehydration were closely followed. As an agent for securing rapid mixing of fluid, I used air which

[4] For the convenience of the reader, the exact technique employed is given below. The modified Bouin fluid is made up as follows:

A.	Picric acid, saturated aqueous solution	75 cc.
	Formol, C. P.	25 cc.
	Glacial acetic acid	10 cc.
B.	Chromic acic, crystals	1.5 grams
	Urea, crystals	2.0 grams

The ingredients under A may be mixed and kept as a stock solution. Just before using the chromic acid and urea should be added, in the order named, and the solution heated to about 38°C.

In operating for the removal of tissue, the fixing fluid is kept in a water-bath on the same table. Immediately on removal, the testis is slit open and a small mass or two of the tubules are cut out with scissors and dropped into the fixing fluid. The tubules are quickly teased apart so that each of them will be exposed to the action of the fixative. Fixation at 38°C. is carried on for about an hour. The tissue is then placed and washed in 5 per cent alcohol. Using the drop method throughout (an ordinary burette is useful), the tissue is brought through the following solutions: 10 per cent alcohol, to which a few drops of a saturated solution (aqueous) of lithium carbonate has been added. It is kept in this solution until most of the picric acid has been removed. Then 50 per cent alcohol is added until the strength of the solution is about 35 per cent. Then a mixture of equal parts of aniline oil and 50 per cent alcohol is dropped in. This is followed by a similar mixture of 70 per cent alcohol and aniline oil. Next, pure aniline oil is added and the tissue cleared in this. The aniline oil is replaced by wintergreen oil. From this it is embedded in paraffin, but the tissue is passed through some six or eight changes in which there is an increasing proportion of paraffin. The time taken for embedding is around three hours.

In the second method, urea is added to ordinary strong Flemming solution to the extent of 0.5 gram per 100 cc. The solution is then chilled with ice to about 4°C. The tubules are teased out in this fluid and kept in it on ice for twenty-four hours. Subsequently it is washed and treated in the ordinary way. From 95 per cent alcohol one may pass to oil of cedar (using the drop method), then xylol, and finally paraffin.

was forced to bubble through the solutions containing the material. After using Flemming, Hance's suggestion of clearing with cedar oil directly from 95 per cent alcohol was followed.

The embryos employed were both unborn and pouch young. Only the cold Flemming method was used for preserving this material.

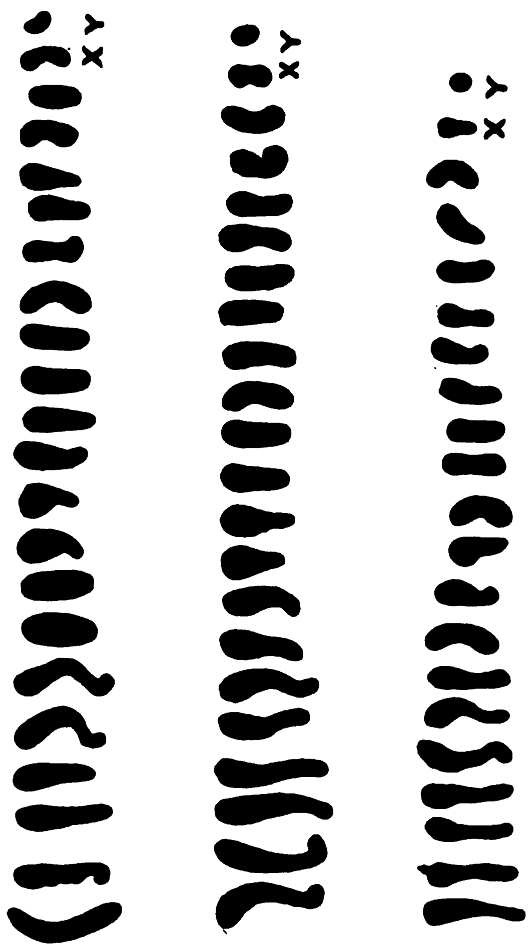
Embedding was carried on in the usual way and sections cut at from 6 to 8μ. Iron-haematoxylin was the method used for staining.

The author is indebted to Dr. Carl Hartman for much of the living material used for the present work, and also for the privilege of examining some of his preparations of polar spindles, which will be found figured on page 36.

SPERMATOGONIAL DIVISIONS

Dividing spermatogonial cells, in equatorial plate view, show 22 chromosomes (figs. 1 to 6, see also somatic divisions, fig. 5, on page 26). Of these 21 are elongated rods, of various sizes, bent or shaped in characteristic ways, and one small rounded chromosome. There are no typical V-shaped chromosomes in the opossum complex. Usually the twenty largest chromosomes form a ring about the two smallest (figs. 1 to 4, also somatic divisions), but occasionally this condition does not obtain (figs. 5 to 6). On inspection of the figures it will be noted that there are a number of pairs of chromosomes, similar in size and shape; and, further, that the two chromosomes lying in the center of the spindle are decidedly smaller than the remaining twenty elements. This fact is important and is shown in all figures (figs. 1, 2, 3, 4, and 6) where there is no great foreshortening of the chromosomes. It is brought out, best, perhaps, by arranging the chromosomes of any spindle in their approximate size relations. In text figure 1 the chromosomes of the cells shown in figures 2, 4, and 6 are copied by the aid of a copy camera lucida, and arranged in approximately the order of their size. It will be seen, first, that the twenty larger chromosomes can all be paired up, with more or less accuracy, and, secondly, that the two smallest chromosomes not only are decidedly smaller, but that they have no

synaptic mates of the same size. These two smallest elements are, as will be shown, the sex-chromosomes, the rounded element being the Y-component and the bent rod the X-component. In



Text fig. 1 Showing the chromosomes of three spermatogonia, arranged in approximately the order of their size. The X- and Y-chromosomes are labeled.

figures 1 to 6 the Y-element appears as a rounded body, very conspicuously smaller than the other chromosomes. The X-component frequently shows one end of the rod larger than the

other (fig. 4) or it may even appear as two unequal egg-shaped chromosomes joined as in figure 1. This inequality in the two ends of the X-element persists throughout maturation.

The autosomes are all more or less rod-like, but in some cases there is a tendency for the inner end of a chromosome to be smaller than the outer; the inner end may even be bent at an angle to the rest of the chromosome.

The cells figured (1 to 6) are taken from three different males; there is no appearance of any fusion of the chromosomes and little overlapping (fig. 1, 2, or 4). In some cells there is a tendency for certain chromosomes to occur together, either lying side by side (fig. 6) or one partly on top of the other (fig. 5). In poorly preserved material such chromosomes would doubtless appear as one element.

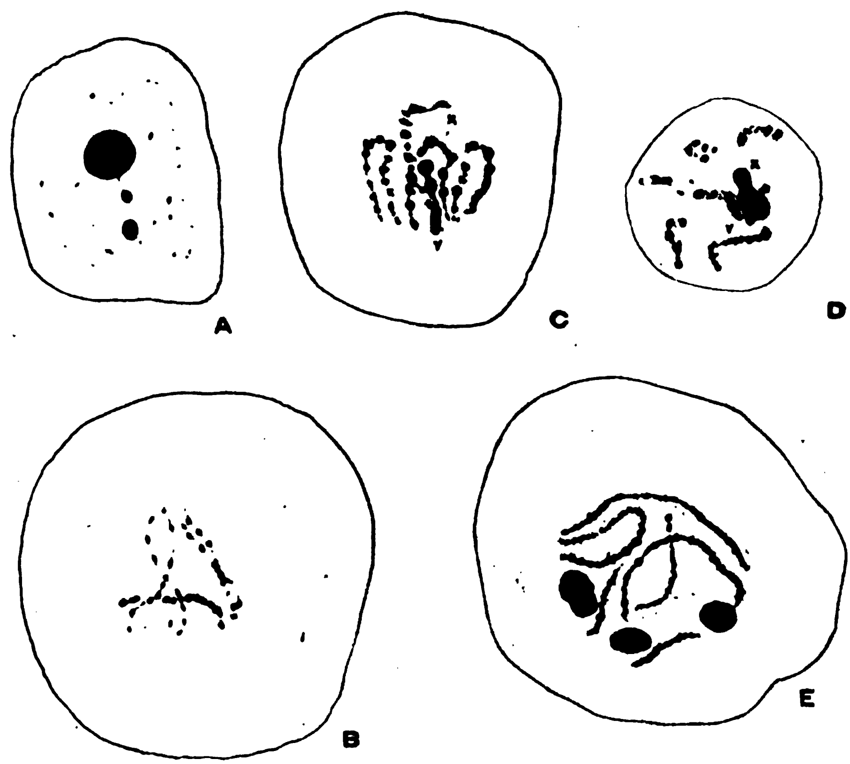
In addition to the counts figured in plate I a great number of other drawings of dividing spermatogonial cells were made by the aid of a camera lucida, and counts then verified. In every case 22 was found to be the number of chromosomes present.

The division of spermatogonia offers no point of especial interest, as it proceeds in the usual way. No lagging behind of any elements has been seen.

FIRST MATURATION DIVISION

The author has made no attempt to follow in minute detail the changes which the chromosomes undergo from the early growth period to the time when they enter the first maturation spindle. Jordan has clearly figured and described the essential facts; my own preparations show stages similar, in the main, to those found by him. A few points illustrated in text figure 2 will be touched upon. The so-called 'diffuse stage' (text fig. 2, A) is characterized by three nucleoli, which lie scattered among the faintly staining chromatin knots. The two smaller nucleoli are unequal in size. With the appearance of the leptotene threads, some deeply staining areas are found in the nucleus, which probably represent one or more of these nucleoli. The pairing of the leptotene threads is seen with almost schematic clearness in the opossum. In text figure 2, B are three pairs

of threads, drawn as they lie in one nucleus. We have three steps of the fusion illustrated here—the threads lying more or less together, a pair of threads in which partial fusion has taken place and a pair of threads in which all but the ends have joined to-



Text fig. 2 Various stages taken from the growth period of the first spermatocytes.

gether. The single leptotene threads are distinctly knotted, and in synapsis these knots join together. There is some contraction (synezesis) of the nuclear content at this time, but it is not very marked. The 'bouquet stage' (text fig. 2, C) is a striking feature of many of my slides. In addition to the thread loops, one

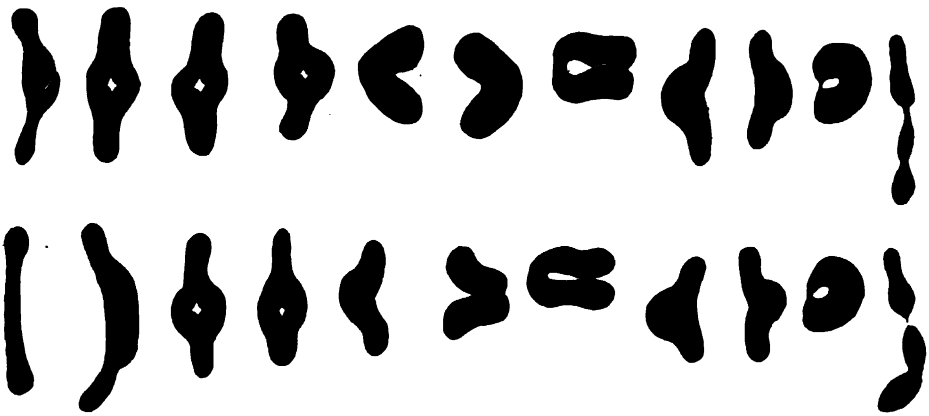
often sees clearly the deeply staining chromatin masses labeled *X* and *Y*. Following this period, the chromatin threads expand until they fill the entire nucleus and the chromosomes become woolly in appearance (text fig. 2, D and E). The whole cell remains in this phase for a considerable time. In early stages (text fig. 2, E) three nucleoli are found, but the homologies of these with those described in the early growth period (text fig. 2, A) are not clear. As this stage advances, however, two of the nucleoli seem to disintegrate, they swell, lose their affinity for the stain, and eventually disappear from view. Some indication of this may be seen in text figure 2, E. The third nucleolus persists and frequently has a form (text fig. 2, D) which suggests that it is made up of the X and Y chromosomes lying side by side. The contraction of the diplotene threads follows the disappearance of the two nucleoli, and the tetrads of the first maturation spindle take on their characteristic forms.

In the first maturation division spindles, there are 11 chromosomes (figs. 7 to 11). It has proved difficult and unsatisfactory to determine the number of chromosomes present from ordinary equatorial plate views. The reason for this is not because of a fusion of the elements, but because of the shapes of the chromosomes themselves, as a glance at the figures will show. In side views of the spindles, particularly just as the tetrads are entering the spindles, and in slides in which destaining has been carried out to an advanced degree, one can usually make out ten typical tetrads and an eleventh chromosome which is tripartite in form, that is, made up of two egg-shaped elements, and a blunt rod joined end to end. This is the X-Y chromosomes complex. (In fig. 10 two of the chromosomes, which lie underneath the rest are drawn out at one side.)

The ten tetrads have such characteristic forms, that after experience one can usually identify all of them and determine which are lacking, in case the spindle has been cut in two. In text figure 3 the typical shapes of these tetrads, as taken from two cells (figs. 7 and 10), are shown. A comparison of the chromosomes figured in text figure 3, with the spindles in figures 7 to 12, will enable the reader to identify most of the elements

for himself. Once one learns to know the shape of the individual tetrads, then polar views of equatorial plates are intelligible and in all complete spindles 11 chromosomes are found.[5]

The history of the sex-chromosome complex is given in the adjoining text figure 4. A close inspection of this complex (labeled *XY*, figs. 7, 10, 12 and text figs. 3 and 4) shows that it is made up apparently of three elements arranged end to end. (Only two spermatogonial chromosomes are involved.) There are two egg-shaped masses, the X-element and a blunt rod which is the Y-chromosome. The egg-shaped mass lying distal to the equatorial plate is somewhat larger than the other similar body.



Text fig. 3 The chromosomes of the first maturation spindle. All are tetrads except the X-Y elements.

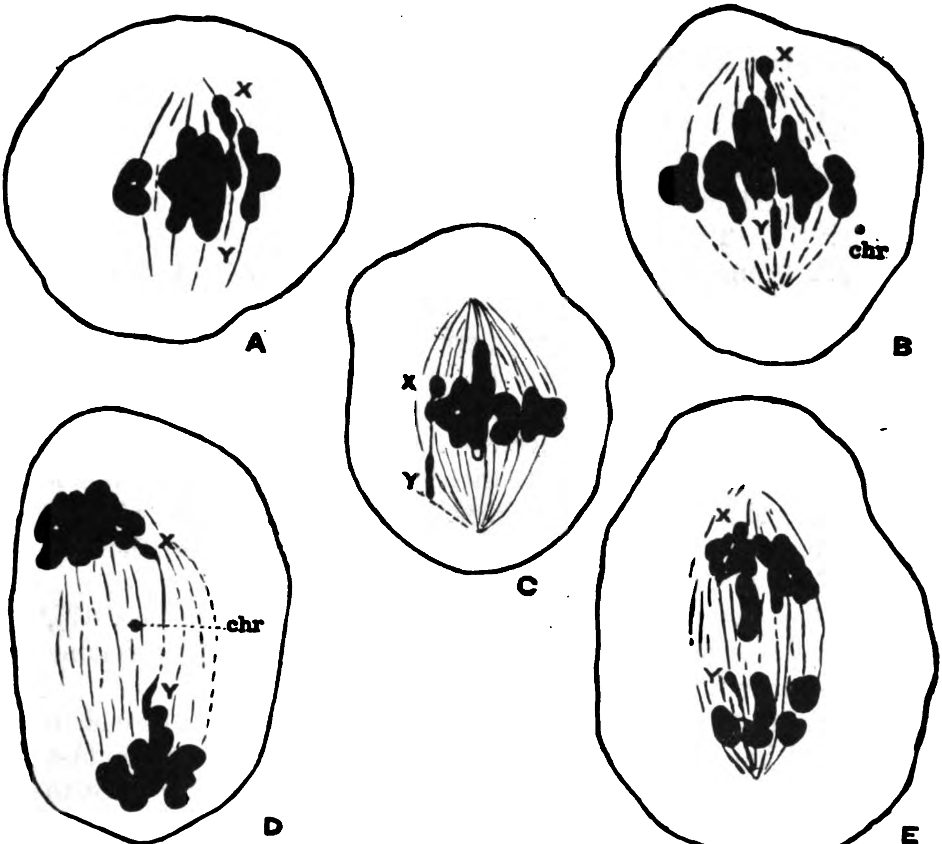
The Y-chromosome may appear as bulged at one end (figs. 7 and 10), but more frequently it is simply an elongated mass of chromatin (text fig, 4, A to E).

The behavior of the X-Y chromosome complex is illustrated in figures 7, 8, 10, 12 and in text fig. 4. The time of division may be early (text fig. 4, A, B, and C) or late when these elements are found lagging behind in the spindle (text fig. 4, D and E). In either case the plane of division is such that the egg-shaped ele-

[5] The fact that mammals have elongated tetrads in the first maturation division (the author has observed them also in the bat and the striped skunk) undoubtedly explains the great difficulty which has been experienced in making counts at this period.

ments, or X-chromosome, go to one pole, while the Y-chromosome goes to the other. No exceptions to this have been noted.

As a result of the first maturation division, the secondary spermatocytes all receive 11 chromosomes, but in half of these cells



Text fig. 4 Showing various stages in the separation of the X- and Y-chromosome elements in the first maturation division.

we have the X-element and in the other half the Y-chromosome is present.

A distinct resting stage follows the first maturation division, as Jordan has described and figured. I fail to find, however, any very striking differences in the nucleoli of such resting cells.

Frequently, in first spermatocyte cells, a chromatoid body is found (labeled *chr.* in fig. 3, text fig. 4, B and D). It usually lies well out of the spindle, and no attempt has been made to follow its history during maturation.

SECOND MATURATION DIVISION

In equatorial plate views all secondary spermatocytes show the presence of 11 chromosomes (figs. 13 and 17); of these the autosomes are all typical bivalent or dumb-bell-shaped elements. The sex-chromosome varies in appearance, depending on whether we are dealing with the X-component (fig. 13) or the Y-component (fig. 17). The X-element usually appears as a quadripartite (fig. 13) and the Y-element as a short rod (fig. 17) sometimes bipartite.

The history of the second spermatocytes is given in figures 13 to 20. In figure 18 we see the X-chromosome as it appears in side views of the spindles, and in figures 16 and 19 we can follow the distribution of this element to the spermatids. It will be noted that each spermatid received a pair of egg-shaped chromosomes which are joined together. Figure 14 is a polar view of one end of a second spermatocyte spindle (telophase) showing the X-chromosome plus 10 autosomes.

When the Y-chromosome is present in the spindle (fig. 17), it divides equationally (figs. 15 and 20), each spermatid receiving a Y-element.

Figures 19 and 20 are two cells, which in the testis lie end to end. They are probably the daughter cells of a single first spermatocyte, as no other cells in a similar stage are close to them. In the one daughter (fig. 19) the X-element is seen dividing and in the other daughter (fig. 20) the Y-chromosome is dividing.

It seems to have been the common experience of a number of workers on mammalian spermatogenesis that in the second spermatocyte there is a tendency of the chromosomes to fuse in pairs, thus bringing about what has been termed by Guyer 'double reduction.' In my slides the fact cannot be denied that some of the chromosomes do fuse in a way which is suggestive

of double reduction. This is especially apt to be the case where the second spermatocyte cells lie deep within the tubule. On the other hand, when the second spermatocyte cells lie near the periphery of the tubule, there is no trace of such a fusion. The chromosomes are as distinct and separate as one could wish.

THE CHROMOSOME NUMBER OF OPOSSUM EMBRYOS

A study of the spermatogenesis has shown that in the male opossum there is a pair of chromosomes which segregate out in the first maturation division and are distributed to the spermatids in a way comparable to the typical X-Y chromosomes of the insects. From a study of the male alone it could not be definitely stated, for the opossum, which was the X-element, or female-producing component, and which the Y-element, or male-producing component. In order to clear up this point and to further verify the spermatogonial chromosome counts, a study of the chromosome complex of opossum embryos was undertaken.

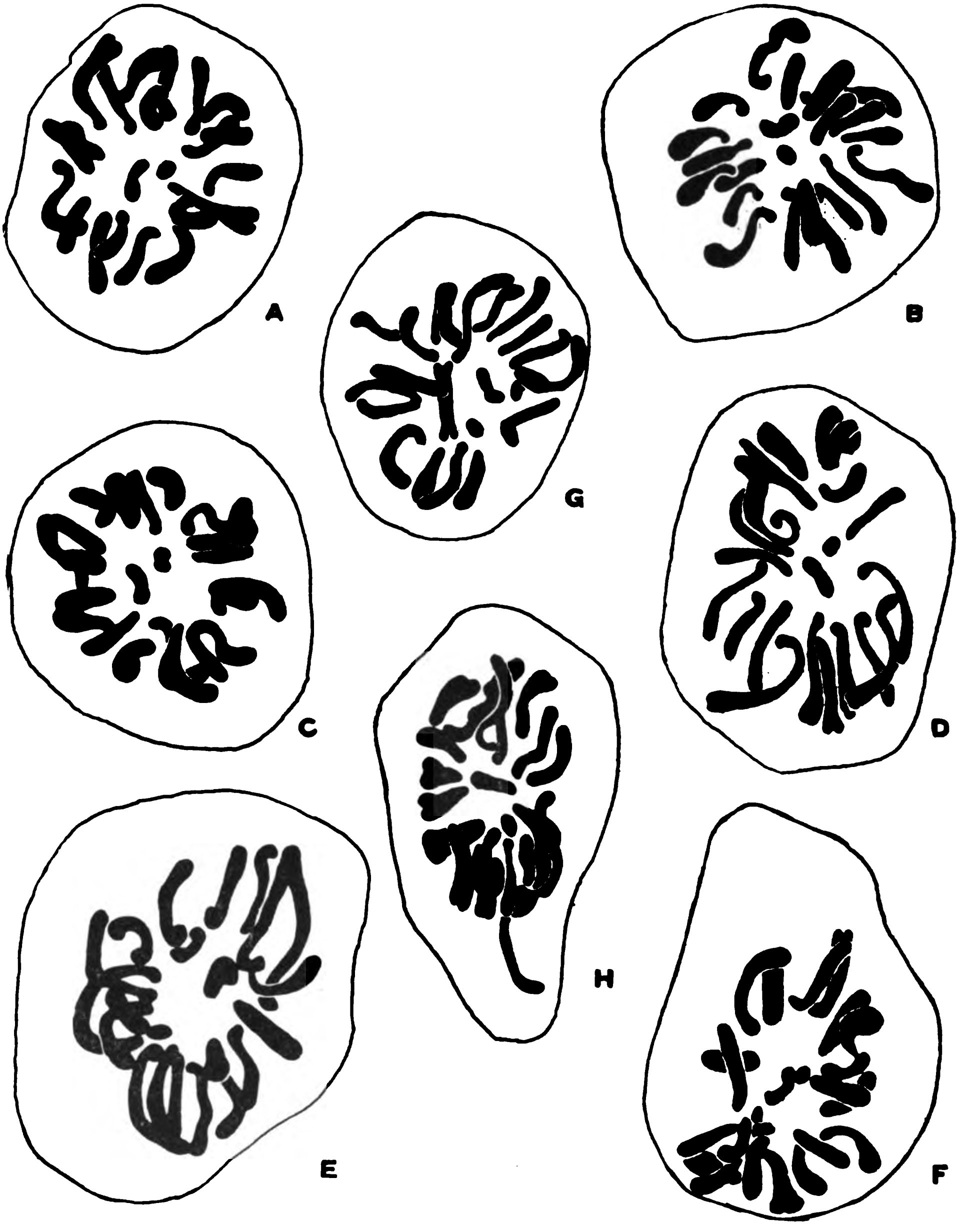
Five very young embryos of unknown sex were used together with three female embryos, whose sex was previously determined by Doctor Hartman. In all cases cold Flemming solution, with urea, was used as the fixative. The dividing cells principally of the nerve cord were used for making counts.

At first the five embryos of unknown sex were studied.

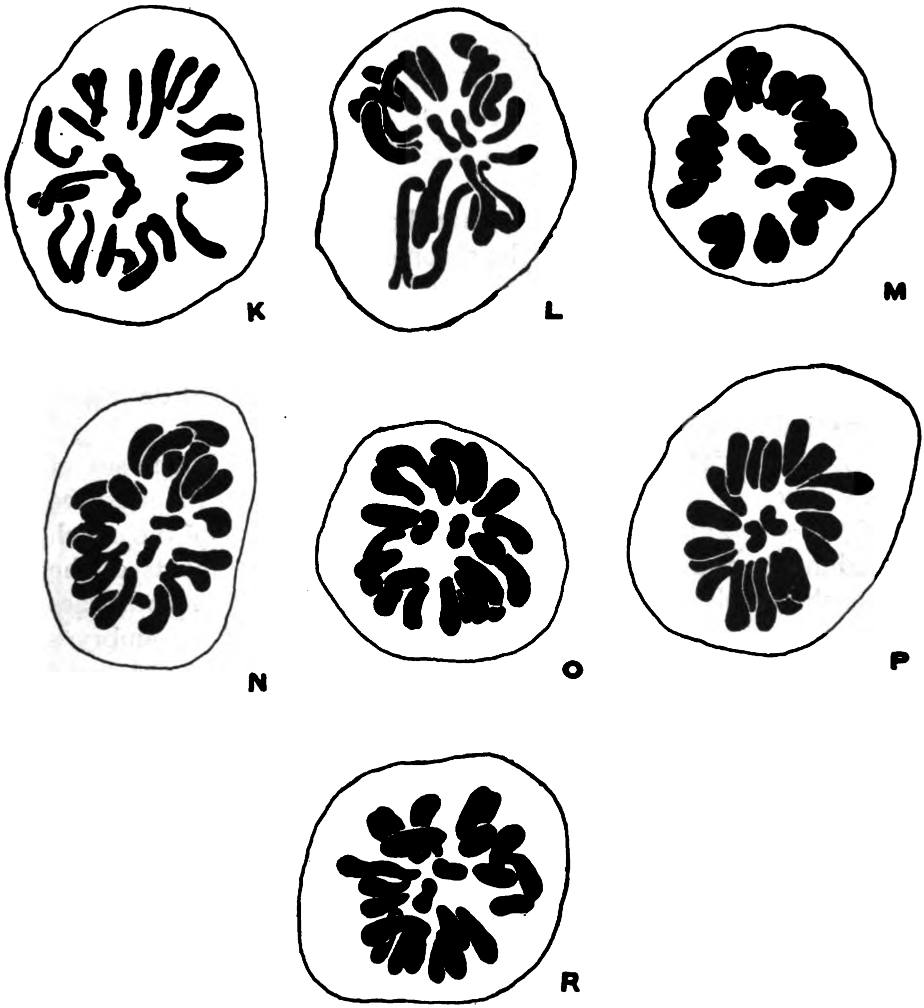
Embryo no. 4 showed the condition illustrated in text figure 5, A and B. There are 22 chromosomes in the spindles, two of these are smaller than the rest and are similar to the small elements found in the spermatogonia. Embryo no. 4 clearly possesses the chromosome complex of a male opossum.

Embryo no. 5 (text fig. 5, C and D) showed, like no. 4, 22 chromosomes, including the small X-Y elements. Again, we are dealing with an embryo clearly possessing the male chromosome complex.

Embryo no. 6 (text fig. 6, K and L) showed consistently 22 chromosomes. The small round chromosome was absent, however, and in its place there is a small elongated element similar to the other sex-chromosome. This condition of the chromo-



Text fig. 5 Equatorial plate views of dividing somatic cells of four male embryos.



Text fig. 6 Equatorial plate views of dividing somatic cells of four female embryos.

somes was found in all cells of this embryo. The absence of the small rounded chromosome and the presence of a second elongated rod was interpreted as meaning that embryo no. 6 was a female with the 2-X condition. This showed, then, that the small elongated rod was the X-component, and on this basis the rounded chromosome of the male was the Y-component.

Embryos nos. 7 and 8 (text fig. 5, E, F and G, H) both turned out to be males.

Thus it will be seen that among the first five embryos studied four were found to possess the chromosome complex of the male while only one showed the female condition. Although the study of the chromosome complex was consistent in that it showed all males to have the X-Y condition and the one female always to have the 2-X condition, a further check was deemed desirable, especially as the material was at hand.

Doctor Hartman has found that he can identify the sex of the pouch young of the opossum soon after birth, by the presence of the scrotal swellings in the male and the absence of the pouch rudiments and, conversely, in the female the rudiments of the pouch are plainly visible and the rudiments of the scrotal sacs are absent. Doctor Hartman selected for me three embryos which he identified as being of female sex. They were preserved in cold Flemming and sectioned.

Text figure 6, M to R, shows the chromosome complex of these three individuals. The figures show that in every case the 2-X condition of the sex-chromosomes is found just as in embryo no. 6 (text fig. 6, K and L) and also that there are 22 chromosomes in all the cells. (Text fig. 6, M and N from embryo no. 10; O and P from embryo no. 11, and R from embryo no. 9.)

In none of the eight embryos did I find any evidence for a fragmentation of the chromosome elements, as Hance did in a similar study of pig embryos. Among the hundreds of cell plates examined, I invariably found 22 chromosomes. In embryos nos, 4, 5, 7, and 8 the X-Y chromosome condition was observed, and in embryos nos 6, 9, 10, and 11 the 2-X condition prevailed.

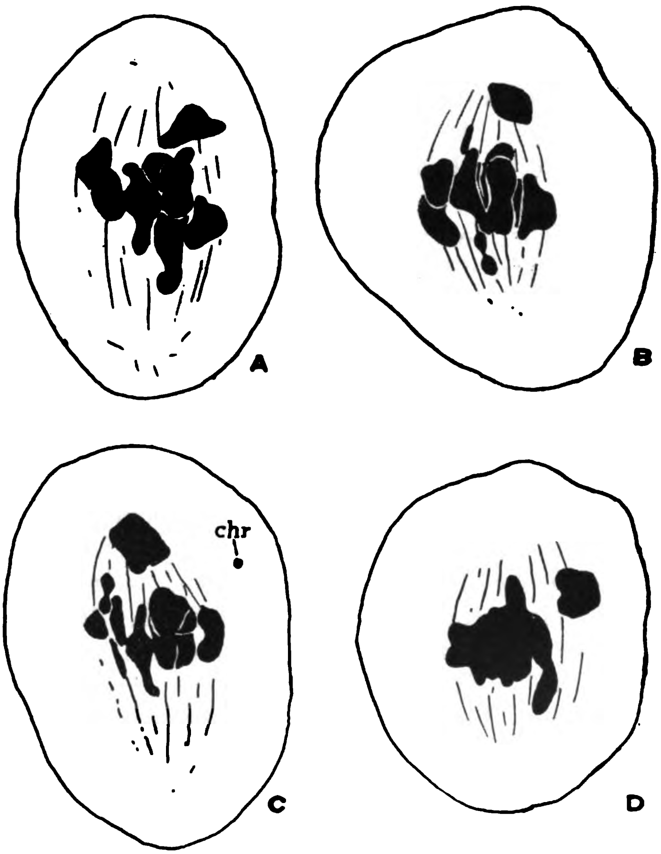
DISCUSSION

1. Sex-determination in the opossum

In the foregoing pages evidence has been presented which seems to demonstrate conclusively that sex-determination in the opossum is of the X-Y type for the male, and not the X-O type, as reported by Jordan. The facts adduced to prove this conclusion are briefly as follows: In the spermatogonia there are 22 chromosomes (figs. 1 to 6). These can all be paired up except the two smallest which have no synaptic mates; these are the X-Y chromosomes. The X-component is a small, somewhat elongated rod, the Y-element is a single rounded chromosome. In the growth period of the first spermatocytes we find a nucleolus which is probably the X-Y components lying side by side (text fig. 2, D). In the first spermatocyte spindles there are 11 chromosomes, showing that all 22 spermatogonial chromosomes have mated (figs. 7 to 11). Of these eleven chromosomes, ten are conspicuous tetrads, while the eleventh is made up of three parts arranged linearly. The figures, however, show that the tripartite chromosome is really the X and Y elements joined end to end. When division occurs the three elements are separated so that the two egg-shaped parts (the X-components) go to one pole, while the single rod-like Y goes to the other. In the secondary spermatocytes we find in part of the cells the X-element alone, and in other cells the Y-chromosome occurs by itself. In both cases the sex-chromosome divides equationally, so that all spermatids receive 11 chromosomes. Half of the spermatids receive 10 autosomes plus the X-chromosome, and half receive 10 autosomes plus the Y-chromosome. As an additional bit of evidence substantiating the facts outlined above, are the dividing cells of opossum embryos, in which we either have the X-Y conditions (males) or the 2-X condition (females). Sex determination for the opossum follows the scheme given below:

Sperm contain:		*Eggs contain:*		
10 autosomes + X,	plus	10 autosomes + X,	=	20 autosomes + 2X (female)
10 autosomes + Y,	plus	10 autosomes + X,	=	20 autosomes + XY (male)

The question now arises, what is the explanation for the bipartite body which Jordan figures, as going undivided to one pole of the first maturation spindle, and which he interpreted as being the X-chromosome? The probable answer is, that what



Text fig. 7 Side views of the first maturation spindle, showing displaced tetrads which would appear as true sex-chromosomes in poorly fixed material. The X-Y chromosomes are visible in two of the spindles.

Jordan saw was either a displaced tetrad or half of a tetrad, the other half remaining in the equatorial plane of the spindle. Such conditions are not rare in my material. In text figure 7, several cells are figured, in which a tetrad has been displaced and appears

to be passing undivided to one pole. That these bodies are false accessory chromosomes is proved by the fact, first, that the X-Y elements (the true sex-chromosomes) can be clearly seen in most cells, and, secondly, that these false accessory chromosomes usually have the typical shapes of tetrads which reveal their true nature. If the preservation of the material were not so good, these tetrads would appear as more or less bipartite, and would doubtless be interpreted as a true accessory chromosome if the X-Y elements were not in prominence.

The cause of this occasional displacement of tetrads, which one observes here and there in the spindles, is probably due to the technique employed (either the sectioning razor or to diffusion currents set up during fixation and subsequent treatment of material) and doubtless does not occur in the living cell.

2. Sex-determination in mammals

The opossum has long been cited as a mammal in which we have the typical X-O type of sex-chromosome for the male. And since the appearance of Jordan's work in 1911 many investigators, reporting on the spermatogenesis of other mammals, have described a body similar in appearance and behavior to the 'accessory chromosome' found by Jordan, and have interpreted it, as Jordan did, as the true sex-chromosome. It may be added that in most cases no attempt was made to work out the complete history of the chromosomes in the way Jordan had done. And yet as the present work seems to prove clearly the true sex-chromosomes of the opossum are of the X-Y type, and that what Jordan saw was probably a displaced tetrad, or possibly half a tetrad which had passed early to one pole, the other half remaining in the equatorial plane of the spindle. Both of these conditions have been found by the author in his opossum material. In view of these facts, it seems that we may, with all fairness, raise the question, whether or not in some of the other mammals the same error of interpretation has not been made; in brief, whether or not the true sex-chromosome has been found for many forms.

Table 1 gives in a condensed form the results of all workers on mammalian spermatogenesis in which the sex-chromosome has been figured and described. (This list is complete to date as far as known to the author.)

A glance at the table will show that both the origin and the form of the sex-chromosome are variable. It is interesting to note that in the guinea-pig, the rabbit, and, one author claims, for man, the X-Y condition has been described, while in all the

TABLE 1

FORM	DIPLOID CHROMOSOME NUMBER	TYPE OF SEX-CHROMOSOME	BEHAVIOR OF SEX-CHROMOSOME	AUTHOR
Homo sapiens	22	X-O	1st div. reduc.	Guyer, '10
Homo sapiens	23–24	X-O	1st div. reduc.	Montgomery, '12
Homo sapiens	47	X-O	1st div. reduc.	Winiwarter, '12
Homo sapiens	24	X-Y	2nd div. reduc.	Wieman, '17
Dog	21	X-O	1st div. reduc.	Malone, '18
Cat, domestic	35	X-O	1st div. reduc.	Winiwarter and Sainmont
Armadillo	31?	X-O	1st div. reduc.	Newman and Patterson, '10
Opossum	17	X-O	1st div. reduc.	Jordan, '11
Guinea-pig	56?	X-Y	1st div. reduc.	Stevens, '11
Rabbit	22	X-Y	1st div. reduc.	Bachhuber, '16
White rat	37	X-O	1st div. reduc.	Allen, '18
House mouse	39	X-O	2nd div. reduc.	Yocum, '17
Horse	37	X-O	1st div. reduc.	Wodsedalek, '14
Pig	18	X-O	1st div. reduc.	Wodsedalek, '13
Cattle	37	X-O	1st div. reduc.	Wodsedalek, '20

rest of the cases we have the X-O type of sex-chromosome. It should be added, however, that a great many investigators have failed to find evidence for sex-chromosomes in many of these mammals for which it is reported.[6]

Unquestionably, the evidence for the existence of the X-O type of sex-chromosome is very complete in some cases, such as in the rat (Allen) or cattle (Wodsedalek), but in many other cases

[6] Mention is made only of the papers in which an accessory chromosome has been described and figured. A complete review of the literature will be found in Ethel Browne Harvey's paper in the Journal of Morphology, vol. 34, 1920.

the evidence is not convincing, and in view of the results obtained for the opossum it is to be desired that the other mammals where the old technique was used should be reworked with the newer methods of Allen and of Hance. Only when this is done shall we be able to accept as settled the question of the sex-chromosome complex of any given mammal.

3. *Double reduction*

Is 'double reduction' of the second spermatocyte chromosomes a process which normally occurs in spermatogenesis, or may we take the term as being synonymous with poor preservation and technique? As far as the opossum goes, and the author suspects this will hold for all vertebrates, it is perfectly certain that a fusion of the chromosome elements is only obtained when poor preservation is in evidence. In case the material is badly fixed one may get not only double, but complete reduction into one chromatin mass!

The following facts are illuminating. In the case of the opossum, it has been found that it was useless to attempt to study any second spermatocyte spindles which did not lie near the periphery of the testicular tubule. If one will observe this precaution, absolutely no evidence for any fusion of the chromosomes will be found. On the other hand, if the second spermatocyte spindles lie deep within the tubule, the chromosomes are all more or less fused, or tangled up so that accurate study of the elements is impossible. It seems clear that the explanation for this is a matter of the quickness with which the cells are killed. If they lie on the periphery of the tubule, then they are quickly killed, and their true form retained. But if they lie deeper, so that penetration goes on slowly, then a great deal of distortion and fusion will take place before fixation is complete.

It is believed, therefore, that, as a normal process, there is no such phenomenon as 'double reduction,' and that when any investigator obtains such a fusion of chromosome elements, he had better take steps to insure more rapid and complete penetration of his fixing fluid.

4. The chromosome number for the male and the female opossum

It has been shown in the foregoing pages that the diploid or somatic number of chromosomes for both the male and the female opossum is 22, and not 17, as stated by Jordan, or 24, as concluded by Hartman. How are the results of this paper to be reconciled with the chromosome counts of Jordan and of Hartman? The following suggestions are offered.

In the case of the males, a careful comparison of Jordan's figures with my own preparations indicates that Jordan had considerable fusion of the chromosomes in his slides. The Y-chromosome seems entirely lacking from all his figures, and the X-component is present in only one or two cells. The main error in counting, however, was in the chromosome ring. The fact has already been pointed out in the early part of this paper that in spermatogonia one frequently finds cells in which two, sometimes four, chromosomes lie close together, or even partially overlap. Unquestionably, with fixing fluids less adapted to vertebrate chromosomes than the newer methods, these chromosomes would fuse or lie so close together that the most competent investigator could not distinguish their separate outlines. Here, it is believed, is the explanation for Jordan's failure to find more than 17 chromosomes in the spermatogonia.

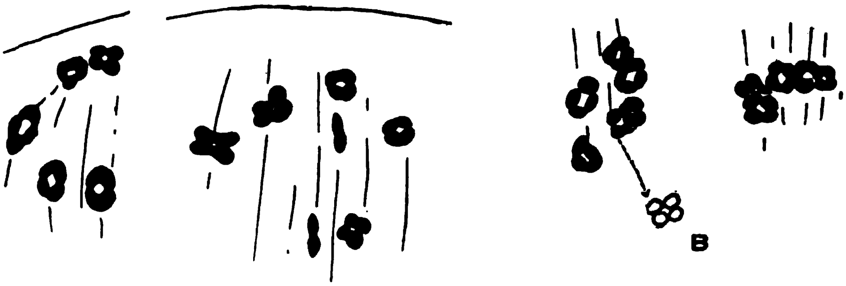
In this connection it is illuminating to consider Hance's ('17) work upon the pig. Wodsedalek ('13) had reported 18 as the spermatogonial number of chromosomes for the pig, while Hance, using cold Flemming solution with urea, found 40 chromosomes in both somatic and germinal cells. Hance made careful computations of the mass of chromatin in Wodsedalek's figures, and then, after making similar computations for his own preparation, he compared the results. He found that, in Wodsedalek's figures with 18 chromosomes, there was approximately the same mass of chromatin as in his cells showing 40 chromosomes. While the author has made no attempt to compare his results in this way with those of Jordan, there is little doubt but that the same mass of chromatin would be found in both cases.

The fusion which masked the true spermatogonial chromosome number in Jordan's preparations is doubtless responsible for his failure to find 11 chromosomes in the first spermatocytes. I found it very difficult to make counts in equatorial plate views of the first spermatocyte spindles until after I had practically learned to recognize each chromosome by its size and shape. To make convincing drawings of such views is very difficult. In side views of the spindles, however, under favorable conditions (early phases of the spindle in cells fully differentiated), it was a simple matter to make accurate counts. In his second spermatocyte cells, Jordan reported 'double reduction' which does not occur at all in the best of my material.

Altogether, it seems clear that Jordan failed to find 22 chromosomes in the male opossum, because there had been a fusion of some of the chromosome elements in his material. This fusion has been universally met with by all investigators on vertebrate germ cells up to, perhaps, the last five years, and it seems very probable that the chromosome numbers for nearly all vertebrates, as they have been reported before this period, are to be accepted with some reservation. The truth is, the old methods of preservation were simply inadequate to handle the vertebrate chromosomes, and it would be grossly unfair to the pioneer workers in this field to regard their work as carelessly or inaccurately done.

The reason Hartman found 12 to be the haploid number of chromosomes is due, as it turns out, to a precocious splitting of one of the tetrads in the second polar spindle of the egg. Figures 13 and 18 of the present paper will show that the X-chromosome splits so early in second spermatocytic division that, if one did not know the origin and fate of the halves, he would interpret them as being two separate chromosomes. And yet, as figures 14, 16, and 19 show, each pole of the cell receives one of these dumb-bell-shaped halves. Before I had an opportunity of examining Doctor Hartman's preparations, I had concluded that probably this is what happened in the eggs where he found 12 chromosomes. In other words, that in such cases Hartman's preparations would show 10 tetrads plus two diads.

Later Doctor Hartman was able to find for me one of his clearest cases of twelve chromosomes in the second polar spindle, and placed the slide at my disposal for study. In text figure 8, A I have drawn the chromosomes of the egg illustrated by Hartman in plate 14, figures 3 and 4, of his paper. There can be absolutely no question of the accuracy of Hartman's count, as there are 12 chromosomes present in this egg. However, on closer examination, it will be seen that 10 of these chromosomes are tetrads, while two of them are bivalent or are diads. (This condition was indicated in Hartman's figures but the magnification of the cell was not sufficient to make the fact clear.) Judging



A

B

Text fig. 8 Showing the chromosomes in the second polar body spindle of two eggs. A. Twelve chromosomes are present in this spindle, of these ten are tetrads and two are diads. This spindle was figured by Hartman ('19) in figure 3 and 4, of plate 14, of his paper. B. Eleven tetrads are present in this spindle. (One of the tetrads is drawn to one side to show its morphology.)

from my experience with the X-chromosome in the second spermatocytic division, these two diads are halves of what should be the X-chromosome tetrad.

As a further check on this conclusion, I selected from Hartman's slides an egg showing 11 chromosomes. This egg is illustrated in text figure 8, B. A glance at the figure will show that all 11 chromosomes are tetrads, and that no diads are present.

It is clear, therefore, that the female opossum has 11 chromosomes (tetrads) in the reduced number, and it would follow from this that the diploid number is 22, as I have found to be the case in the somatic cells of female embryos.

SUMMARY

There are 22 chromosomes in the spermatogonia of the opossum. The two smallest of these constitute a typical X-Y sex-chromosome complex, which can be followed through the growth period of the first spermatocyte. In the first maturation spindle there are 11 chromosomes—10 tetrads and the X-Y chromosome. The X and Y components segregate from each other in this division so that the secondary spermatocytes contain either 10 autosomes plus X, or 10 autosomes plus Y. There are 11 chromosomes in all secondary spermatocytes, of these one is either the X-component or the Y-component. In either case the sex chromosome divides equationally. Half the sperm carry the X- and half carry the Y-chromosome.

A study of dividing somatic cells of embryos showed that in both male and female embryos 22 chromosomes were present. In the males the X-Y condition was found, while in the females the 2-X condition existed.

It is concluded that Jordan's failure to find more than 17 chromosomes in the spermatogonia was due to faulty fixation. The 'accessory chromosome' which he described was not the true sex-chromosome, but probably a displaced tetrad. Hartman's error in count was due to the precocious splitting of the X-chromosome tetrad in the polar spindles.

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PLATES

EXPLANATION OF PLATES

All of the figures represent a magnification of about 3,000 diameters as they are reproduced, except text figures 2, 4, and 7, which were drawn at about 3,000 diameters and reduced by $\frac{3}{5}$. The camera lucida was used for drawing together with a $\frac{1}{12}$ oil immersion (B. & L.) and a no. 15 ocular.

PLATE 1

EXPLANATION OF FIGURES

1 to 6 are equatorial plate views of dividing spermatogonia. Fig. 1, from male no. 1, figs. 3 and 5 from male no. 2, and figs. 2, 4, and 6 from male no. 3.

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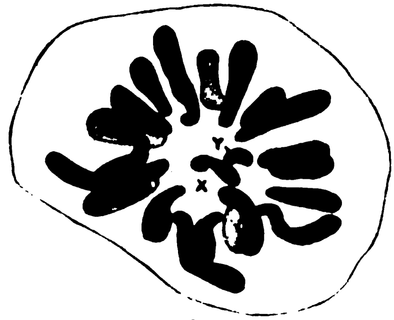

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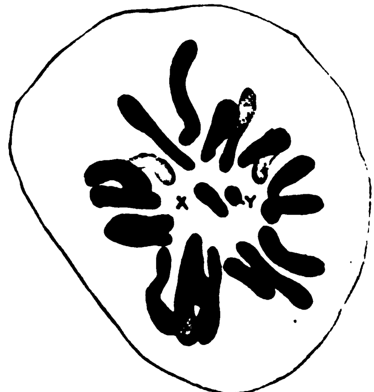

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PLATE 2

EXPLANATION OF FIGURES

7 Side view of first maturation spindle, 11 chromosomes present.

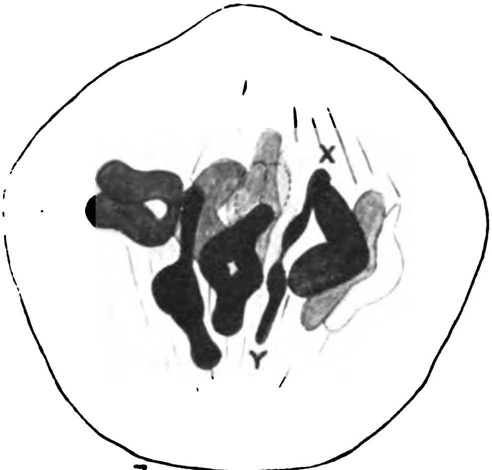
8 Same as above, with 11 chromosomes present. The X- and Y-components have separated.

9 Same as above. Two of the tetrads have been displaced.

10 Same as above. Two of the chromosomes which in the spindle under the others have been drawn out to one side in order to show their shapes.

11 Same as above, showing 11 chromosomes. Only the X-component is visible.

12 Same as above, showing with especial clearness the morphology of the X-Y.chromosome complex. Not all chromosomes are present in this spindle.



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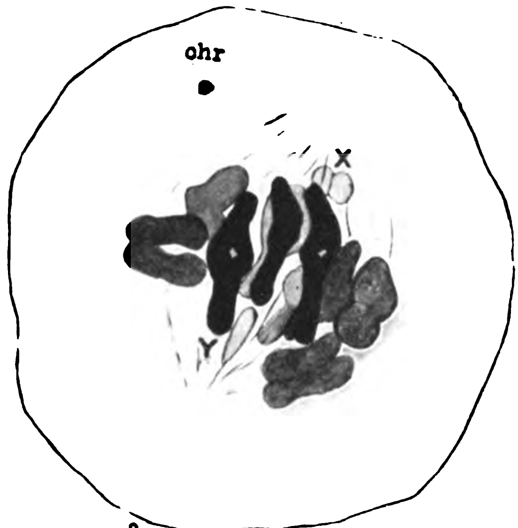

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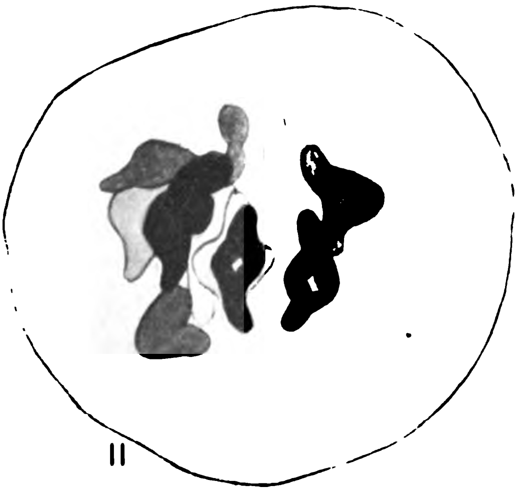

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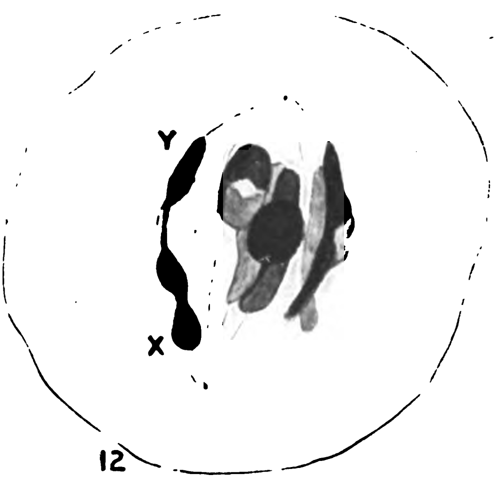

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PLATE 3

EXPLANATION OF FIGURES

13 Equatorial plate view of dividing second spermatocyte. Note the quadripartite form of the X-chromosome. Eleven chromosomes present in this spindle.

14 End view of late telophase stage, showing chromosomes at one end of the spindle. Eleven chromosomes are seen, including the bilobed X-chromosome.

15 Late anaphase of second spermatocytic division showing the Y-chromosome at each pole of the cell. There are 11 chromosomes clearly visible at the lower pole of the cell.

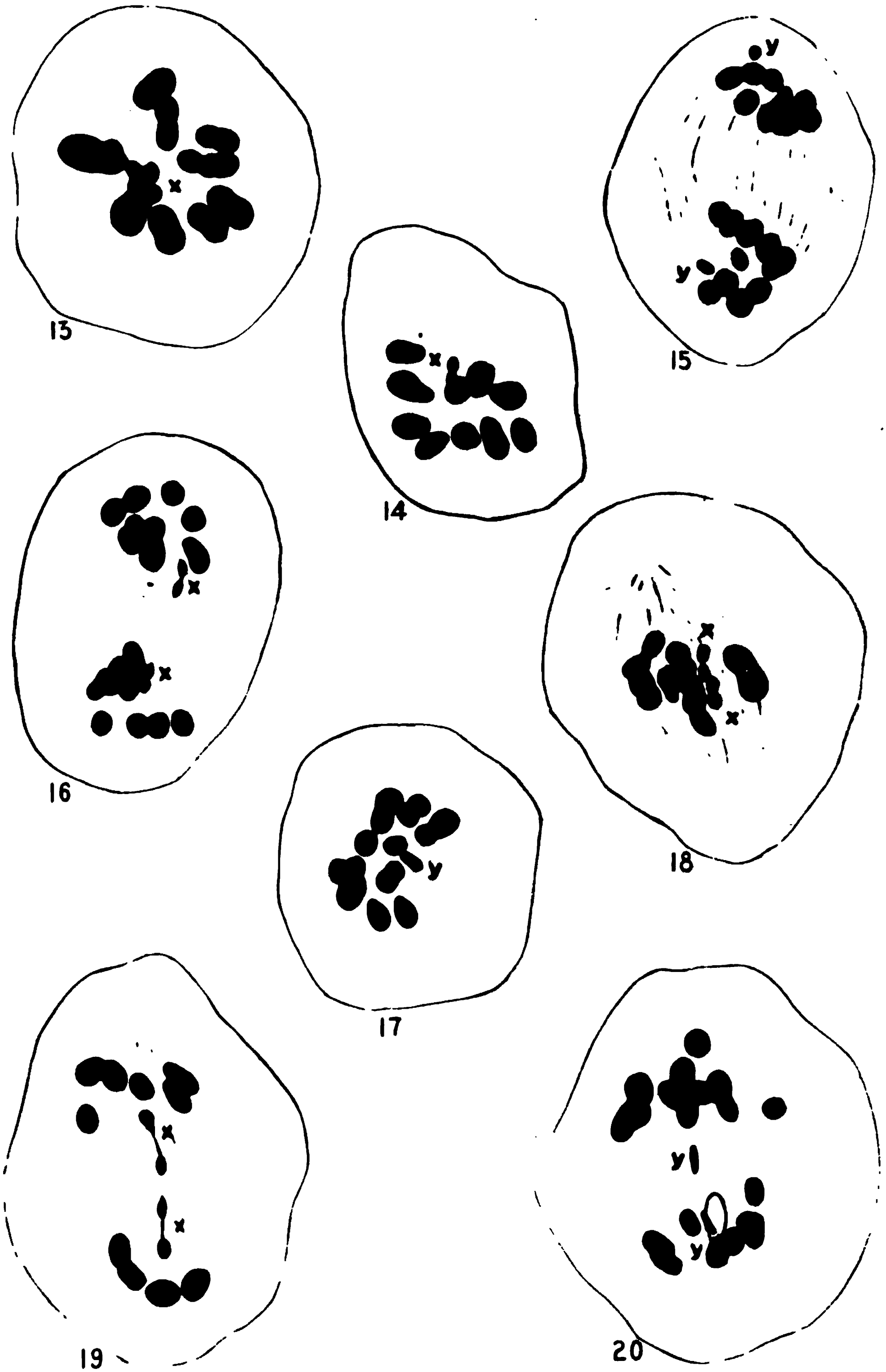
16 Same as 15, except that the X-chromosome is seen.

17 Equatorial plate view of dividing second spermatocyte, showing the presence of the Y-chromosome.

18 Side view of second spermatocyte spindle, showing the precocious separation of the halves of the X-chromosome.

19 Late anaphase of second maturation spindle, showing the presence of the X-chromosome.

20 Same as above, except that the Y-chromosome is seen dividing. The cells represented in figs. 19 and 20 are probably daughter cells of a single spermatocyte, as explained in text.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Resumen por el autor, Joseph H. Bodine

Los efectos de la luz y la decapitación sobre la cantidad de CO_2 producida por ciertos ortopteros.

Los datos consignados en el presente trabajo demuestran que cuando se barnizan de negro los ojos de los saltamontes suprimiendo de este modo la iluminación se observan cambios definidos en la tensión o tono de los músculos y que estos están asociados con cambios visibles en la cantidad de anhidrido carbónico producido por el organismo. Los individuos decapitados también producen menos anhidrido carbónico.

Translation by José F. Nonides
Cornell Medical College, New York

AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
BY THE BIBLIOGRAPHIC SERVICE, DECEMBER 27

THE EFFECT OF LIGHT AND DECAPITATION ON THE RATE OF CO_2 OUTPUT OF CERTAIN ORTHOPTERA

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THREE FIGURES

As a result of the work of various investigators, particularly of Moleschott,[1] J. Loeb,[2] and C. Ewald,[3] it has been pointed out that the effects of light on the respiratory exchange of organisms are rather variable. In some cases definite increases in the respiratory exchange have been noted, while in others no direct effects have been detected. More recent investigations, especially those of Holmes,[4] Garrey,[5] and J. Loeb,[6] on the tropic responses of organisms to light, have definitely shown that the primary effect of light consists in changes in the tension or tonus of muscles. It is the intention of this paper to show that such changes in muscle tension or tonus in an insect are associated with corresponding detectable changes in the rate of carbon-dioxide output of the organism.

The choice of suitable material and a method delicate enough to detect slight differences in CO_2 output are of prime importance in such an investigation. Grasshoppers have been chosen because of the ease in handling, their relatively small size, the fact that they show rather definite tropic responses, and, lastly, that by proper handling their body movements can be practically eliminated during experiments. Nymphs or adults of the following species of grasshoppers were used: Chortophaga australior, Chortophaga viridifasciata, Melanoplus differentialis, and Dichromorpha viridis. All animals were kept in the laboratory and fed grass, lettuce, etc.—the same as those used for other experimental work. Carbon-dioxide determinations were made by the indicator method described by Dr. N. H. Jacobs,[7] of this

laboratory. With this method the CO_2 output was ascertained by noting the time required by the animal to produce definite amounts of CO_2. In blackening the eyes of the animals asphalt varnish was used. This varnish was found to give off no substances which affected the indicator solution.

It has been repeatedly pointed out, as the result of experiments to explain the functions of the nervous system of insects, that the brain is the seat of peripheral nerves, the center for inhibiting reflex movements, and for controlling the tonus of the muscles (Bethe[8]). More recent investigations, and especially those on the functions of the brain of the grasshopper (Ewing[9]), have definitely shown that the brain controls the tonus of the muscles. It was further shown by this author that neither the supra-oesophageal ganglia or brain of the grasshopper nor the sub-oesophageal ganglion was the center for respiratory movements. Each ganglion of the thoracic and abdominal ventral cord was found to be the center for respiratory movements and reflex actions of the segment and the appendages to which it belonged. It was also pointed out that not only the whole abdomen, but different segments of it continued their respiratory activity when severed from the body. Loeb[6] has shown that light acting on the eyes of an animal produces definite effects upon the tension or tonus of the muscles. Lyon[10] and Garrey,[5] by blackening both eyes of insects, were also able to show a decrease in the neuromuscular tonus which was normally maintained reflexly by the action of light on the eyes.

Both the experiments on decapitation and blackening of the eyes have been repeated on grasshoppers by the author, and results which agree with those of the above-mentioned investigators have been obtained. It was thought, however, that some quantitative measure of the effect of light and of the brain on muscle tension or tonus could be gotten by estimations of the CO_2 output of the organism.

BLACKENING BOTH EYES

When one eye or parts of one or both eyes of an animal are blackened, the characteristic changes in posture, etc., are produced; but slight disturbances to the animal due to the partial blackening cause it to try to remove the varnish with its front legs. Such movements are not easily eliminated, and hence no satisfactory results on the CO_2 determinations are possible. However, when both eyes are completely blackened, the animal makes no attempt to remove the varnish, but remains motionless.

Carbon-dioxide determinations on the animal before blackening its eyes are, with proper handling and manipulation, rather easily and accurately made. In the experiments herein reported at least two or three separate determinations, in which there were no detectable body movements, were made and the average rate of CO_2 output for the normal animal then obtained. No difference in movements of the animal in the normal and eye-blackened condition could be detected, so results are not, to any appreciable extent at least, modified by CO_2 produced as the result of body movements.

Table 1, in which are listed results of some ten typical experiments, shows the time taken to produce the same amount of CO_2 by an animal when normal and with eyes blackened. In almost every case a marked decrease in the rate of CO_2 output is noted. Those animals, in which the decrease in rate of CO_2 output was not so marked with eyes blackened, when decapitated also showed a comparatively small decrease in rate. These slight decreases in rate of CO_2 output in certain animals can doubtless be attributed to the physiological condition of the particular organism.

Figure 1 shows graphically the average decrease in rate of CO_2 output for fifty individuals.

To show that this decrease in rate of CO_2 output was due to the effects of blackening the eyes, and that after removal of the varnish the animal assumed its normal conditions, the following experiment is cited. Individuals were taken, the normal rates of CO_2 output determined, and then the eyes blackened. The

TABLE 1

Showing the rate of CO_2 output of grasshoppers when normal, with eyes blackened, and when decapitated

TEMPERATURE	SEX	SPECIES	STAGE OF GROWTH	BODY WEIGHT	TIME IN MINUTES TO PRODUCE SAME AMOUNT OF CO_2		
					Normal	Eyes blackened	Decapitated
°C.				grams			
23	♂	C. australior	Adult	0.2140	10.0, 6.5, 7.0 Av. 7.8	11.5, 12.5, 14.5 Av. 12.8	22.0, 22.0, 24.0 Av. 22.6
23	♂	C. australior	Adult	0.1750	13.5, 11.5, 12.5 Av. 12.5	17.5, 18.0, 20.0 Av. 18.5	18.0, 20.0, 20.0 Av. 19.3
23	♂	C. australior	Adult	0.2240	6.0, 5.5, 5.5 Av. 5.6	9.5, 7.0, 8.0, 8.0 Av. 8.1	12.0, 11.0, 11.0 Av. 11.3
21	♀	C. australior	Adult	0.3520	4.0, 3.5, 4.0 Av. 3.8	5.5, 5.5, 5.5, 5.5 Av. 5.5	7.0, 9.0, 7.0, 9.0 Av. 8.0
19	♂	C. australior	Adult	0.2250	7.5, 9.0, 8.0 Av. 8.1	13.0, 14.0, 13.0 Av. 13.3	16.5, 16.0, 16.5 Av. 16.3

27	♀	C. australior	Nymph	0.2450	7.0, 8.0, 8.0 Av. *7.6*	10.5, 10.5, 10.5 Av. *10.5*	13.0, 11.5, 12.0 Av. *12.1*
25	♀	C. viridifasciata	Nymph	0.2870	6.5, 6.5, 6.5 Av. *6.5*	8.5, 7.5, 8.5 Av. *8.1*	12.0, 11.0, 12.0 Av. *11.6*
23	♂	M. differentialis	Nymph	0.0600	17.5, 16.5 Av. *17.0*	17.0, 17.0 Av. *17.0*	18.5, 18.0 Av. *18.2*
22	♂	M. differentialis	Nymph	0.0565	20.0, 20.0 Av. *20.0*	26.0, 24.0 Av. *25.0*	26.0, 26.0 Av. *26.0*
20	♀	M. differentialis	Nymph	0.1070	15.0, 17.5 Av. *16.2*	20.5, 20.0 Av. *20.2*	22.0, 22.0 Av. *22.0*

rate of CO_2 output of the animals with blackened eyes dropped characteristically and remained so. After several hours the varnish on the eyes became dried and brittle and could easily be scraped off with fine forceps, leaving the eyes again exposed. Immediately after removal of the varnish the rate of CO_2 output increased considerably, being slightly higher than the original normal rate. This increased rate, however, lasted only a relatively short time, when the normal rate was resumed. Figure 2 shows graphically the results of such an experiment.

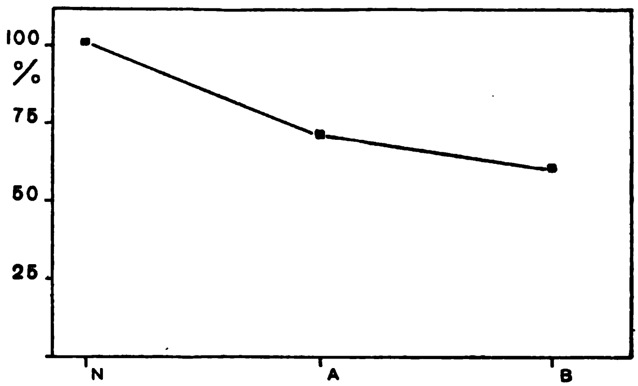

Fig. 1 Curve showing the effect of blackening the eyes and of decapitation on the rate of CO_2 output. Based on average of fifty individuals. Ordinates represent the rate of CO_2 output. The normal rate (which is taken as 100 per cent in each case) corresponds to the time to produce a definite amount of CO_2 (the same amount in any experiment). Points on abscissas indicate different experimental condition of animals, *N*, normal animals; *A*, animals with eyes blackened; *B*, decapitated animals.

That the decrease in rate of CO_2 output was a permanent condition as long as the eyes were blackened is shown by the following. Several individuals of the same species and kept under as near the same conditions as regards food, etc., as possible were taken and their rates of CO_2 output determined. Half the individuals were then subjected to the eye-blackening process and the characteristic decrease in rate of CO_2 output obtained. The remaining individuals were kept as controls. All the animals were starved for twenty-four hours under the same laboratory

conditions and CO_2 determinations were then made. It was found that the animals with blackened eyes respired at a much lower rate than the normal individuals. The varnish of the blackened-eyed animals was next removed and a marked increase in rate of CO_2 output resulted. Figure 3 shows graphically the results of such an experiment.

We may summarize the results of the above experiments by stating that a marked decrease in rate of CO_2 output results from a cutting off of the illumination of the eyes and that this effect is a permanent one so long as the eyes are blackened.

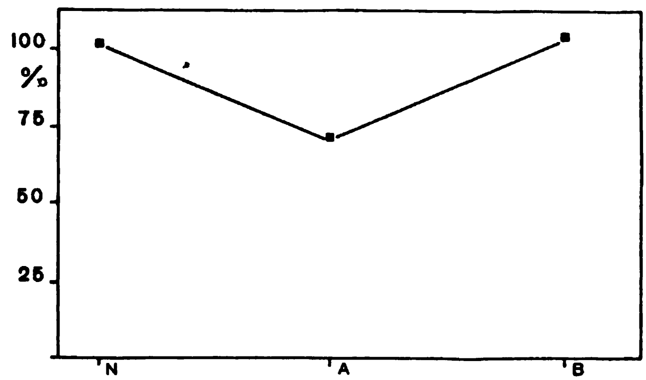

Fig. 2 Curve showing the effect of blackening the eyes and of the removal of the varnish from the eyes on the rate of CO_2 output. Based on average of six individuals. Points on abscissas, *N*, normal animals; *A*, animals with eyes blackened; *B*, animals from whose eyes varnish has been removed. Ordinates same as in figure 1. For further description see text.

DECAPITATION

Since it has been pointed out that the brain of insects exerts a marked effect on the tonus of muscles, but does not control the respiratory movements, it was thought that by decapitation a check on the previous results on blackening the eyes could be made.

Decapitated animals were used three-quarters to one hour after the operations, so that any effects of the operation would not interfere with the experiments. The posture, etc., of the animal after decapitation were much the same as when the eyes were

blackened. Both animals whose eyes had and had not been previously blackened were used, but no differences in response were noted. Table 1 and figure 1 show that a decrease in the rate of CO_2 output was always associated with decapitation. The degree of this decrease varies slightly for different animals, and it is usually lower than that for individuals with blackened eyes. When decapitated, the amount of actual respiring tissue taken off with the head is rather large and can account for much of the decrease in rate below that for blackened-eyed individuals. The

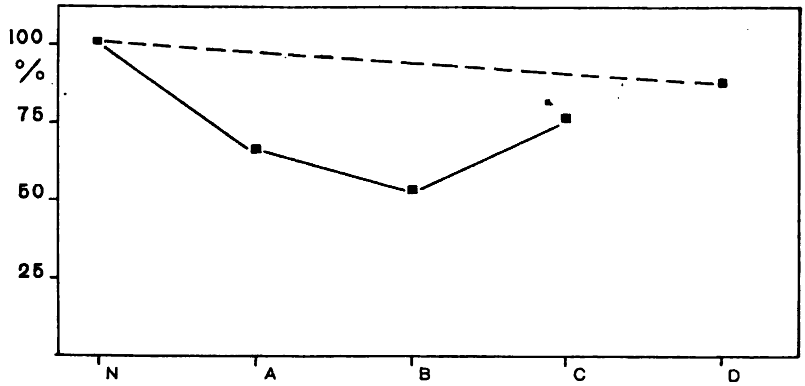

Fig. 3 Curves showing the effect of blackening the eyes, shortly after the operation, twenty-four hours after, and the removal of the varnish after twenty-four hours on the rate of CO_2 output. Based on average of six individuals. Solid lines for experimental animals. Broken lines for normal animals. Points on abscissas, *N*, normal animals at start; *A*, animals with eyes blackened—at once; *B*, animals with eyes blackened—after twenty-four hours; *C*, animals from whose eyes varnish was removed—after twenty-four hours; *D*, normal animals after twenty-four hours. Ordinates same as in figure 1. For further description see text.

operation itself also doubtless exerts some effect. The combined effects of these two factors undoubtedly account for much of this decreased rate, and as a matter of fact it has been found that when the head is put into the respiration tube with the decapitated animal the decrease in the rate of CO_2 output is strikingly similar to that observed for blackened-eyed individuals.

The above results then show that decapitation results in a marked decrease in rate of CO_2 output not greatly different in magnitude from that observed for blackened-eyed individuals.

SUMMARY

The action of light on the eyes of an animal (like the grasshopper), affecting the tonus of muscles, is associated with a decrease in the rate of CO_2 output of the organism. A similar decrease in rate of CO_2 output is also found in decapitated individuals.

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Resumen por el autor, Dwight E. Minnich

La sensibilidad química de los tarsos de la mariposa Pyrameis atalanta Linn.

En la mariposa Pyrameis atalanta Linn. cada uno de los cuatro tarsos ambulatorios posee quimoreceptores de contacto cuya estimulación apropiada produce una respuesta en la forma de una extensión de la proboscis. Esta respuesta sin embargo varía algo con la naturaleza química del estímulo y con la condición de la nutrición del animal. Un estudio intenso de estas variaciones ha hecho posible establecer con certeza algunas de las substancias que la mariposa puede distinguir mediante sus tarsos. De los cuatro estímulos probados, tres de ellos, a saber, el agua destilada, I M de sacarosa y 2 M de NaCl son claramente distinguidos por la mariposa. El cuarto, M/10 de cloruro de quinina es claramente distinguido del agua destilada y de la solución 2M NaCl y probablemente también de la IM de sacarosa. Puesto que los órganos tarsales son quimoreceptores de contacto que sirven para distinguir el agua y el alimento normal tal como la sacarosa, pueden propiamente considerarse como órganos del gusto.

Translation by José F. Nonides
Cornell Medical College, New York

AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
BY THE BIBLIOGRAPHIC SERVICE, DECEMBER 27

THE CHEMICAL SENSITIVITY OF THE TARSI OF THE RED ADMIRAL BUTTERFLY, PYRAMEIS ATALANTA LINN.

DWIGHT E. MINNICH

Department of Animal Biology, University of Minnesota

THREE FIGURES

CONTENTS

INTRODUCTION

In a previous paper (Minnich, '21) I showed that the tarsi of the butterfly, Pyrameis atalanta Linn., are sensitive to contact with certain substances in solution, and hence must possess contact chemoreceptors. In the same paper I also presented some preliminary data indicating what kinds of substances the animals were thus enabled to discriminate. It is the purpose of the present paper to give a further account of the qualitative range of sensitivity of these tarsal organs.

In the present work I have employed the same general methods as before, with two exceptions. First, instead of using both captured specimens and those hatched in the laboratory, only butterflies hatched in the laboratory have been employed. The complete adult history of every specimen was thus a known and controlled one. Second, a few animals have been studied, under varying nutritional conditions, over a long period of time, rather than a number of animals, in the same nutritional condition, for a short period of time. Indeed, the data to be presented have been obtained from but eight specimens, and chiefly from four of these. These four butterflies, however, were kept under very close observation for 19, 27, 29, and 30 days, respectively.

GENERAL METHODS

The experiments were carried out chiefly during the month of July. The laboratory was a basement room of northwest exposure, but direct sunlight was kept out by white-cloth shades at the west windows. Ventilation was accomplished entirely from the interior of the building, so that it was possible to avoid sudden and pronounced fluctuations of temperature. During the entire period of experimentation the extremes of temperature registered in the laboratory were 21°C. and 25.9°C. For the great majority of days, however, the temperature ranged from 22° to 24°, the variation for the day being less than 1°. Ventilation from the inside of the building also insured a more uniform condition of humidity than outside ventilation would have allowed. Every precaution was thus taken to maintain as great constancy of the general laboratory environment as possible.

The specimens employed were all exceptionally large and perfect. After hatching, they were housed in large cages (0.9m. x 0.4m. x 0.4m.) consisting of a light wooden framework covered with mosquito bar. Excepting periods of starvation, which were practiced from time to time during experiments, the animals remained in excellent condition. In one or two instances, near the end of an experiment, one leg of a specimen became more or less stiff and functionless as far as locomotion was concerned. Also the wings, in addition to being clipped slightly for purposes of indentification, became somewhat frayed and considerably rubbed in their outer portions, due to the effects of the holder in repeated trials. But as far as I was able to observe, these slight mutilations in nowise affected the reactions which were being studied. In general, the hairy proximal portions of the wings and the rest of the body remained in almost perfect condition—much more so than is the case with animals in a state of nature. Thus butterflies caught in the field frequently have lost portions of the labial palpi, but of the eight animals in my experiments not one suffered such a mutilation.

Even in their most vigorous states, however, the butterflies flew but little. It was not that they were unable to fly, but that they merely did not. Neither did they creep much, usually

remaining at or near the place where they were released upon completion of a trial. This inactivity was doubtless due in a large measure to the effect of the spring clothes-pin holder on the wings in repeated trials, to continuous confinement, and to the constancy of the environmental conditions, viz., temperature, humidity, and light.

As in my previous work, the results of the present experiments have been obtained entirely through a study of the conditions which effect an extension of the proboscis. I have described this response in considerable detail (Minnich, '21, p. 178), so that it will be sufficient merely to mention the essential features here. In the unstimulated animal, the proboscis remains compactly coiled against the head, but upon appropriate stimulation it is extended and begins to probe the substrate. Not infrequently, however, a given stimulus fails to elicit a complete extension, producing only a partial extension followed by a subsequent recoil. Indeed, the partial extension may be so slight that the compact coil of the proboscis merely exhibits a jerk or two with no further sign of activity. Between such slight reactions and complete extension, all gradations may be observed.

It is clear that all extensions of the proboscis, whatever their degree, represent responses. But it is equally clear that these responses differ in intensity. To measure such differences is difficult. Nevertheless, I have endeavored to approximate a measurement by weighting all responses in which the proboscis was uncoiled less than one-half at 0.5, and all in which it was uncoiled one-half or over at 1. In figure 1, no. 1, the proboscis is shown as it normally appears in the unstimulated animal. In the same figure, nos. 2 and 3 illustrate responses which would be weighted at 0.5, while nos. 4 and 5 show responses which would be weighted at 1. As a matter of fact, the case illustrated in no. 4 is virtually never encountered, for the proboscis is rarely extended one-half or more of its length without being completely extended. This scheme for measuring the intensity of the proboscis response, therefore, virtually amounts to weighting small partial extensions at 0.5 and complete extensions at 1. I shall employ this scheme in all comparative statements, in order that

evaluations of groups of responses may be compared as well as the mere numbers of responses.

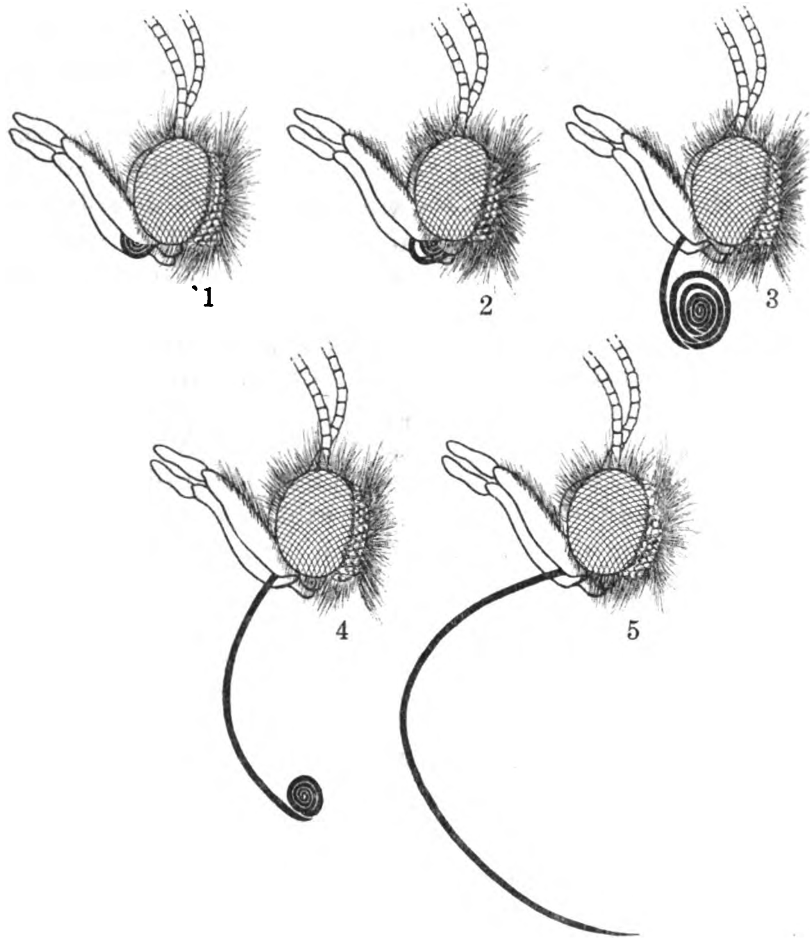


Fig. 1 Diagrams showing the proboscis in the unextended condition and in various stages of extension: 1, unextended; 2 and 3, partial extensions which would be weighted at 0. 5; 4, partial extension which would be weighted at 1; 5, complete extension, which would be weighted at 1.

In the present experiments every trial was immediately preceded by a preliminary trial held under the same conditions. The purpose of the preliminary trial was fourfold: 1) to overcome

the death feint, although this reaction is virtually negligible in Pyrameis; 2) to eliminate the immediate effects of mechanical manipulation incident to the trial; 3) to accustom the animal to the grip of the holder on the wings, and, 4) to make certain that there was no sign of proboscis extension in the absence of the chemical stimulus to be applied. The last-mentioned point is of particular importance, for extensions of the proboscis were occasionally observed when no external chemical stimulus was apparent. Responses of this sort were most frequently observed in individuals which, through prolonged starvation, had become extremely sensitive. Most of these responses were of slight magnitude, although some consisted of complete extensions. The following examples will illustrate the cases in point.

In all trials butterflies were manipulated by means of a spring clothes-pin in which the wings were firmly held. The most simple method of placing an animal in the holder was often by direct use of the hands. This involved grasping the wings, and later the thorax and wing bases with the fingers. On grasping the wings with the fingers, as the specimen was pulled off the wall of the cage where it had been resting, there was occasionally a slight jerk of the proboscis coil. Again, while the butterfly was being held by the fingers in one position or another, further incomplete or, in rare instances, complete extensions of the proboscis were noted. Because of these occasional responses, direct contact between the hands and the body of the animal was avoided whenever possible. But even when the holder was applied directly to the wings, there were one or two instances of response. The stimuli effecting these responses cannot be postulated with certainty. I am inclined to believe that distance and contact chemical stimuli from the hands were chiefly responsible although it is possible that in conditions of extreme sensitivity mechanical stimuli may also exert some influence.

In several instances a butterfly which had remained undisturbed in its cage fifteen minutes or more was found exhibiting either a partial or complete extension of the proboscis. The previous trial having been completed fifteen to twenty minutes before, it was impossible to interpret such a case as a persisting

response. Internal stimuli due to prolonged inanition, or external chemical stimuli of an adventitious sort, too dilute for my detection, may perhaps explain these responses, though a certain statement concerning them is not possible.

Without multiplying examples, the above are sufficient to illustrate the behavior in question. One of the chief purposes of the preliminary trial was to detect responses of this sort and thus avoid an occasional misinterpretation. If there was but one barely visible jerk of the proboscis coil as the animal was pulled off the wall of the cage, and no further sign of extension during the preliminary trial, experimentation was continued. If, however, there was any more significant movement of the proboscis during the preliminary procedure, experimentation was discontinued and not resumed for at least fifteen minutes. Usually there was no evidence of response in the second preliminary, but in case there was, experimentation was again discontinued for a minimum period of fifteen minutes.

The maximum duration of all trials, preliminary and final, unless otherwise stated, was one minute. Failure to observe any visible movement of the proboscis during this period constituted a 'no response.' If the proboscis was partially extended early in a trial, the trial was continued, to ascertain whether complete extension would result. If the proboscis was completely extended, the trial was immediately terminated, and the animal returned to its cage. Trials thus lasted one minute or less, depending upon the response.

The chemical stimuli employed consisted of distilled water, and three aqueous solutions, viz., 1M saccharose, 2M NaCl, and M/10 quinine hydrochloride. The saccharose and quinine hydrochloride were USP quality; the sodium chloride, CP quality. The solutes being non-volatile, the distance stimulus afforded by each of the solutions was identical with that afforded by distilled water, viz., water vapor. In other words, the four stimuli used could be distinguished, if distinguished at all, only through direct contact.

With the exception of quinine hydrochloride, the stimuli selected were substances frequently encountered by Pyrameis in

its natural environment. The importance of water in this connection is too obvious to require comment. As for saccharose, it is one of the chief organic compounds present in the substances on which this species feeds, viz., fruit juices, exuding sap, and nectar. Thus apple juice is very attractive to these animals, and, if available, may constitute one of their chief foods in the autumn. In orchards where fallen apples litter the ground, I have observed great numbers of the butterflies feeding, just prior to hibernation. According to Browne ('99, pp. 9 and 10) the flesh of the average ripe apple contains 4 per cent of saccharose, this being, with the exception of water (84 per cent) and invert sugar (8 per cent), the only substance constituting more than 1 per cent of the total composition. Sodium chloride was selected as a common inorganic salt encountered by butterflies in surface waters. It is well known that many species of lepidoptera congregate about drying pools of water, and pools which have been contaminated with urine or manure seem particularly attractive. In such situations, NaCl is present in considerable quantity.

Saccharose and sodium chloride, in addition to the fact that they are frequently encountered by Pyrameis in its natural habitat, happen also to be substances which afford adequate stimuli for two of the four primary taste sensations in man, viz., sweet and salt. Quinine hydrochloride was chosen for experimentation, because it affords the adequate stimulus for a third of the human taste sensations, viz., bitter. With Pütter ('11, p. 608) I agree that there is not the slightest reason to suppose that a substance which affects the human taste organs in a certain way will affect the taste organs of a lower animal in the same way. It was not to ascertain whether Pyrameis could distinguish bitter that quinine hydrochloride was chosen, but rather to discover whether this salt, which produces such a bitter and disagreeable sensation when applied to the human tongue, would produce or fail to produce a reaction in the butterfly.

EXPERIMENTS WITH LOCAL STIMULATION OF INDIVIDUAL TARSI

Three butterflies were kept for five days after hatching without access to food or water. At the close of this period, they were subjected to a series of trials in which various chemical stimuli were applied locally to individual tarsi. The stimuli were applied on small cotton swabs ca. 1 cm. long and 1 to 2 mm. in diameter, the swab consisting of a bit of absorbent cotton wound on the end of a dissecting needle. Care was exercised to keep the cotton chemically clean while being handled. As a further precaution, swabs used to test the effect of dry cotton alone were heated prior to each experiment, in order to drive off any excess moisture.

The butterfly to be tested was placed in a holder (fig. 2) with the four ambulatory feet resting on a small platform of wire screen. In this position, the specimen was closely observed for one minute, this constituting the preliminary trial. In no case was any sign of response observed. Immediately following the preliminary trial, a dry cotton swab was applied to the ventral surface of the distal end of the ambulatory tarsus which it was desired to test. Except for a very few partial extensions this also failed to elicit any response. The dry swab was then replaced by a swab saturated with distilled water, and to this the animal almost invariably responded. Upon completion of the trial with distilled water, the specimen was returned to its cage for a minimum interval of fifteen minutes, after which another tarsus was tested in the same manner. This procedure was continued until each of the four ambulatory tarsi had been tested twice.

On completing the trials described above, the butterfly was placed on absorbent cotton saturated with distilled water, whereupon the proboscis was immediately extended. In this situation—presumably drinking in water continuously—it was allowed to remain as long as it would. After two or three minutes, however, the proboscis was recoiled, and the animal either crept away or was removed. Some minutes later, it was replaced on the wet cotton, and thus given a second opportunity to drink, but in no case was there any response.

After access to water, the butterfly was allowed to rest for a minimum interval of fifteen minutes, whereupon trials were resumed. The specimen was again placed in the holder with its four feet upon the screen platform and carefully observed for one minute. As before, however, there was never any indication of response during this preliminary trial. Next, the dry cotton

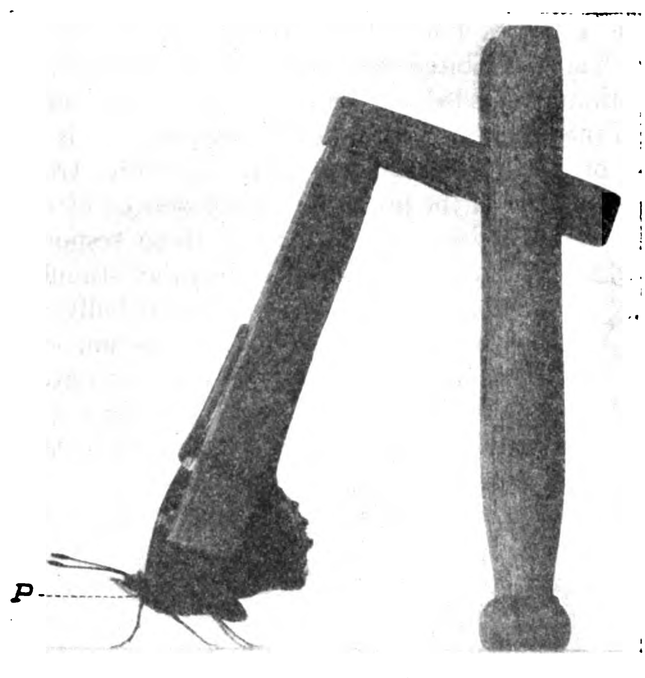


Fig. 2 Photograph showing holder employed in experiments on stimulation of individual tarsi. Note the appearance of the proboscis, *P*, in the unstimulated animal.

swab was applied to the tarsus, but this now failed to elicit even a single partial extension of the proboscis. The dry swab was followed by a swab saturated with distilled water. Prior to the administration of water, this had produced a very high per cent of response. It now failed to produce a response in nearly every trial. An additional stimulus was, therefore, employed, viz., a swab saturated with a 1M saccharose solution. To this stimulus

there was but one failure to respond. On completion of the trial with the sugar solution, the tarsus which had been tested was carefully rinsed by immersing in distilled water, and the butterfly was returned to its cage. Fifteen minutes later another tarsus was tested in the same manner, and so on, until each of the ambulatory tarsi had again been tested twice.

The data obtained from the above experiment are presented in table 1. It will be noted that specimens, both before and after access to water, exhibited essentially the same behavior toward the tactile stimuli afforded by the metal of the wire screen and the cotton of the dry swab, viz., failed to respond. It is true that in the case of the starved animals there were three trials in which partial extensions of the proboscis were produced by contact with dry cotton. Possible explanations of these responses will be discussed later. In comparison with the other stimuli employed, however, the response to dry cotton was virtually zero. With the cotton soaked in distilled water, the situation was very different. Before the animal was allowed access to water, this form of stimulus was 87.5 per cent efficient in producing a response, whereas after access to water, it was but 4.2 per cent efficient. Yet after this virtual disappearance of the response to water, a 1M saccharose solution was still 91.7 per cent efficient in evoking a response.

As noted above, a dry cotton swab applied to the tarsus occasionally elicited a slight response. Precisely what was the effective stimulus afforded by the cotton? Two possibilities may be suggested: first, the mere contact (pressure) of the cotton; second, the hygroscopic water present on the cotton fibers. The fact that the few slight responses observed were in starved animals and that these responses disappeared after access to water lends support to the latter suggestion. A final statement, however, as to the effective stimulus in these responses is not possible, at least not from the present data.

Returning to a consideration of table 1, the following facts are brought out clearly by the data there presented. First, contact (pressure) stimuli alone, such as those afforded by wire screen or dry cotton, when applied to the tarsi have little or no effect in

TABLE 1

Showing the effects of different stimuli on individual tarsi

(1) ANIMAL NUMBER	A BUTTERFLIES 5 DAYS OLD WHICH HAD RECEIVED NEITHER FOOD NOR WATER									B SAME BUTTERFLIES AS IN A, AFTER ADMINISTRATION OF DISTILLED WATER											
	(2) No swab in contact with tarsi			(3) Dry swab in contact with one tarsus			(4) Distilled water swab in contact with one tarsus			(5) No swab in contact with tarsi			(6) Dry swab in contact with one tarsus			(7) Distilled water swab in contact with one tarsus			(8) 1 M saccharose swab in contact with one tarsus		
	No response	Partial extensions	Complete extension	No response	Partial extensions	Complete extension	No response	Partial extensions	Complete extension	No response	Partial extensions	Complete extension	No response	Partial extensions	Complete extension	No response	Partial extensions	Complete extension	No response	Partial extensions	Complete extension
32	8	0	0	8	0	0	0	1	7	8	0	0	8	0	0	8	0	0	0	1	7
33	8	0	0	6	2	0	1	2	5	8	0	0	8	0	0	7	1	0	1	1	6
34	8	0	0	7	1	0	0	1	7	8	0	0	8	0	0	7	1	0	0	0	8
Totals	24	0	0	21	3	0	1	4	19	24	0	0	24	0	0	22	2	0	1	2	21
Total weighted response		0			1.5			21			0			0			1			22	
Per cent of effectiveness[1]		0			6.3			87.5			0			0			4.2			91.7	

[1] The per cent of effectiveness is calculated by dividing the total weight of responses obtained, as defined on page 109, by the total weight which would have been obtained had there been a complete extension of the proboscis in every trial. Thus, in column 3, three responses are listed. Since each represents only a small partial extension, their total weight is 3 × .5 or 1.5. The total weight, had there been a complete extension in every trial, would have been 24. 1.5 ÷ 24 = 6.3 per cent.

producing an extension of the proboscis. Second, the same stimuli plus certain chemical stimuli will effect an extension of the proboscis. Pyrameis must, therefore, discriminate through its tarsi the presence of the chemical stimuli. Third, under certain conditions, distilled water is one chemical stimulus which is very efficient in evoking a response. Fourth, one condition which determines the responsiveness to water is the degree of inanition with respect to water. Satiety with respect to water inhibits the responsiveness thereto. This response may, therefore, be controlled, approaching 100 per cent or 0 per cent, according as the animal is or is not allowed access to water. Finally, although Pyrameis fails to respond to water in a condition of satiety with respect to the same, it, nevertheless, continues to respond vigorously to a 1M saccharose solution. The butterfly must, therefore, be able through its tarsi to distinguish sharply between water and an aqueous solution of a non-volatile substance such as saccharose.

EXPERIMENTS WITH SIMULTANEOUS STIMULATION OF ALL AMBULATORY TARSI

Because of the mechanical difficulties involved in stimulating individual tarsi, a more satisfactory method of experimentation is to allow all the ambulatory tarsi to come in contact with the stimulus at the same time. Experiments using this method were, therefore, more generally employed. In these experiments the wings of the butterfly were held in a spring clothes-pin, which was manipulated by the hand. Upon removal from the cage, the butterfly was first subjected to a preliminary trial of thirty seconds, in which the feet rested on clean filter-paper. This trial was carried out within 3 to 5 cm. of the place where the subsequent trial with a given chemical stimulus was to be made, so that the environment in the two trials was practically identical. During the trial, the butterfly was gently lifted and let down again at intervals of ten seconds, in order that it might become thoroughly accustomed to this sort of manipulation.

If there was no significant response during the preliminary trial, as was usually the case, the butterfly was lifted from the

filter-paper, across the few intervening centimeters, and set down with the feet in contact with a thin layer of cotton, contained in a Syracuse watch-glass and saturated with the solution to be tested. If the animal failed to respond promptly, various slight manipulations were employed to make certain that the ventral surface of every tarsus was afforded ample contact with the stimulus. Thus, if there was no response during the first few seconds of the trial, a slight, even pressure was given the holder, thereby causing the distal portions of one or more tarsi to press more firmly against the cotton and thus become immersed in the solution. If still there was no evidence of response after twenty to thirty seconds, the holder was turned slightly, forcing the butterfly to shift the position of some of its legs. And if both the above measures proved ineffective, toward the end of the trial the animal was occasionally lifted gently and let down again.

At the close of each trial the butterfly was placed over a watch-glass containing distilled water, and the tarsi were thoroughly rinsed. This was necessary in order to prevent contamination of the stimulating substance in one trial by adhering material from previous trials. If the proboscis remained extended after the trial, care was exercised to prevent the animals from drinking at this time. After the legs had been well rinsed, the butterfly was placed on clean filter-paper for a moment to absorb the excess moisture, and was then returned to its cage.

In the above manner, butterflies were tested with distilled water and solutions of 1M saccharose, 2M sodium chloride, and M/10 quinine hydrochloride, four trials being made daily with each of these substances. The order in which the four stimuli were employed was varied from time to time and a minimum rest period of fifteen minutes was allowed between consecutive trials.

The responses of each butterfly were studied under three nutritional conditions: first, a condition in which the animal was receiving neither food nor water; second, a condition in which it was receiving water only, and, third, a condition in which it was receiving both food and water. The general plan was the following. Immediately upon hatching, the specimen was placed

in a cage where it was kept without food or water. After three days, trials were begun with all four substances and were continued until the butterfly gave 100 per cent response to distilled water; that is, complete extension of the proboscis in each of the four trials of the day. In a few cases the experiment was continued for a day or so beyond this point before making any change in the nutritional conditions. In general, however, this was not possible because of the growing weakness of the specimen and the danger of its death.

When the experiment had reached the stage described, at the conclusion of the trials for the day the butterfly was placed on absorbent cotton saturated with distilled water and allowed to drink all it would. On the following morning it usually appeared quite revived. Before resuming trials, however, the animal was again given an opportunity to drink. If it failed to respond to the water, I several times forced an extension of the proboscis by touching one of the tarsi with sugar solution. Thus while the animal could not be compelled to drink, it could be compelled to bring the proboscis in contact with water. In this manner it was offered water in the morning, one hour before trials were begun, and in the evening, immediately after trials were concluded.

The butterfly was continued on this water diet until it again became so weak that further trials were impossible, whereupon experimentation was discontinued for the remainder of the day, and 1M saccharose was administered. Of this solution the animal always imbibed freely, and it was not until the abdomen was greatly distended that it ceased to feed. On the following morning it would again appear quite restored, and trials were resumed. The saccharose diet was continued for 3 to 4 days, administrations being made twice daily as in the case of water.

Following the period of saccharose diet, the butterfly was again kept without water or food until the response to water rose to 100 per cent. Then followed a period of water diet, and when this became insufficient, the sugar solution was again administered. In other words, the three nutritional states described above were repeated.

In continuing periods of starvation as long as possible, the risk of death became very great, and of eight specimens employed, six died in the course of the experiment. The longevity of these individuals was 8, 8, 9, 10, 19, and 27 days, respectively. In all these cases death was most probably due either directly or indirectly to the effects of starvation. In several of the shorter-lived specimens, I might have saved them had I appreciated the gravity of their condition soon enough, for animals in a state of complete collapse may sometimes be resuscitated in a surprisingly short time by the administration of 1M saccharose. Two specimens, however, survived the entire experiment, and several days after their last trials appeared vigorous in every way. They were killed for subsequent morphological study, having been under observation for twenty-nine and thirty days, respectively. Of the eight animals, I shall present the data from the four longest-lived only. The data from the four shorter-lived individuals are less complete and show nothing not shown by the others.

The observations on the four butterflies which survived longest are presented graphically in figure 3. The data for each animal numbered, respectively, 11, 12, 13, and 22, are presented in the form of four curves, each of which represents the responsiveness to a single substance. In these curves, the total weight of daily response, as defined on page 59, is plotted against age in days, the nutritional state for each day being indicated. An examination of the four curves for any one animal shows, with one exception, viz., no. 22, that no two coincide. In other words, there were differences of response to the different stimuli. It should be borne in mind at the outset of this discussion that the fact that a butterfly responds identically to two substances does not necessarily mean that it fails to distinguish them. This may merely indicate a positive response to both substances. On the other hand, differences of response do not necessarily show discrimination, unless they are differences of pronounced nature and regular occurrence. Are the differences noted above such as to indicate discrimination of the various stimuli or not? In order to answer this question, let us examine and compare the responses with respect to: first, relation to nutritional condition; second,

intensity, as indicated by the total amount of response produced, and, third, time aspects, viz., latent period and period of execution.

Let us take first the response to distilled water. This response was characterized by its close relationship to the nutritional condition of the animal. As shown above, in the experiments on stimulation of individual tarsi, table 1, the responsiveness to water could be increased or decreased by preventing or allowing access to water. The same results were obtained when all four tarsi were stimulated simultaneously. The curves of response to water in figure 3 show in every instance that with continued starvation the response finally rose to 100 per cent, while directly after water was administered it dropped to 0 per cent, where it generally remained as long as water or an aqueous solution was accessible. In other words, the response to water depended directly on the nutritional state of the animal. In this respect it was absolutely unique, for in no other response was any intelligible relationship to nutritional condition evident. The response to distilled water is thus sharply differentiated at the outset from the other responses studied. Consequently, we may omit it from further consideration, confining our attention solely to a comparison of the responses to the three solutions.

A survey of the curves of response to 1M saccharose (fig. 3) shows that each of the four butterflies responded to this solution in every trial, irrespective of nutritional condition. My notes show that during periods of inanition the response often became more persistent, lasting for some minutes after the trial had been completed. But even during periods when 1M saccharose was being fed twice a day, there was never a single failure to respond. Thus, a butterfly which had ceased to feed and had crept away, if replaced on the cotton soaked with the solution, immediately responded anew. And, if after ceasing to feed, the specimen remained on the cotton without creeping away, mere seizure of the wings or other slight agitation was usually sufficient to induce a fresh response. The response to 1M saccharose was thus entirely independent of the varying nutritional conditions of the animal.

Not only did the specimens always respond to 1M saccharose, but the extension of the proboscis was in every case a complete one. The total weighted response of all four animals under all conditions was, therefore, 100 per cent. Furthermore, the responses were rapidly executed. As shown in table 2, the average time required for this solution to produce a complete extension of the proboscis was between 1.7 and 3.4 seconds. The response to 1M saccharose was thus characterized by absolute constancy under all nutritional conditions, by maximum intensity, and by rapidity of execution. These facts all indicate that this stimulus must be a very powerful one. Indeed, it was by far the most effective of the stimuli tested.

TABLE 2

Showing the average time in seconds required by different stimuli to effect a complete extension of the proboscis

ANIMAL NUMBER	1 M SACCHAROSE		2 M SODIUM CHLORIDE		DISTILLED WATER		M/10 QUININE HYDROCHLORIDE	
	Number of trials	Average time	Number of trials	Average time	Number of trials	Average time	Number of trials	Average time
11	80	3.4	35	12.7	19	12.5	76	25.4
12	60	3.2	37	5.4	13	16.7	29	43.7
13	90	1.7	30	4.2	16	4.5	86	10.9
22	68	1.8	52	1.7	10	4.3	68	6.1

Unlike 1M saccharose, the responsiveness to 2M NaCl was characterized by extreme variability. One has only to examine the curves in figure 3 to become convinced of this fact. Take, for example, specimen 11. During the first three days of its life, no trials were made. On the 4th, 5th, and 6th days, the responses to this solution were 0. On the 7th, 8th, and 9th days, there was a variable number of responses for each day. On the 10th day the number of responses again fell to 0. On the 11th day it rose to 100 per cent, where it remained until the 16th day. The 16th, 17th, and 19th days (no observations were made on the 18th) the responsiveness was a little less than 100 per cent. On the 20th day it dropped to 0, only to rise to 100 per cent the 21st day, and again fall to 0 the following day, where it remained for the last two days of experimentation. The responses of this

specimen were thus extremely variable, and specimens 12, 13, and 22 showed essentially similar conditions. A careful study of each of these cases fails to show any apparent relationship between the nutritional condition of the animal and its response to the stimulus under consideration. Doubtless this response is very definitely determined, but the determining conditions are not evident from the present data. For the present, therefore, the outstanding characteristic of the response to 2M NaCl is its variability. It may be added that this variability is very great not only from one specimen to another, but also from time to time in the same specimen.

The variability of response to 2M NaCl means, of course, that the total amount of response to this substance was much less than to 1M saccharose. Considering the weighted responses of all four specimens collectively, the sodium chloride produced but 51.6 per cent response, as compared with 100 per cent for the sugar solution. On comparing the curves of the two responses, we find some days when specimens responded indistinguishably to both stimuli. But we also find days when there was a clear-cut difference of response. With every specimen there were periods, ranging from two to seven days, during which the animal gave 100 per cent response to 1M saccharose solution, and yet failed to evince even the slightest indication of response to 2M NaCl. The only plausible explanation of these facts is that the animal discriminated clearly between the stimuli. The fact that it responded at times to both is in nowise incompatible with this interpretation, while the fact that at other times it responded 100 per cent to one and 0 per cent to the other can hardly be explained in any other way. Clearly, therefore, the tarsi enable Pyrameis to distinguish a 1M saccharose solution from a 2M sodium-chloride solution.

There remains for consideration the response to quinine hydrochloride. In certain specimens, for example, no. 12 and to a slight extent no. 11, the curve of response to this substance (fig. 3) also shows some variation from day to day, though much less than with sodium chloride. No. 22, however, responded with a complete extension of the proboscis in every trial, and no. 13

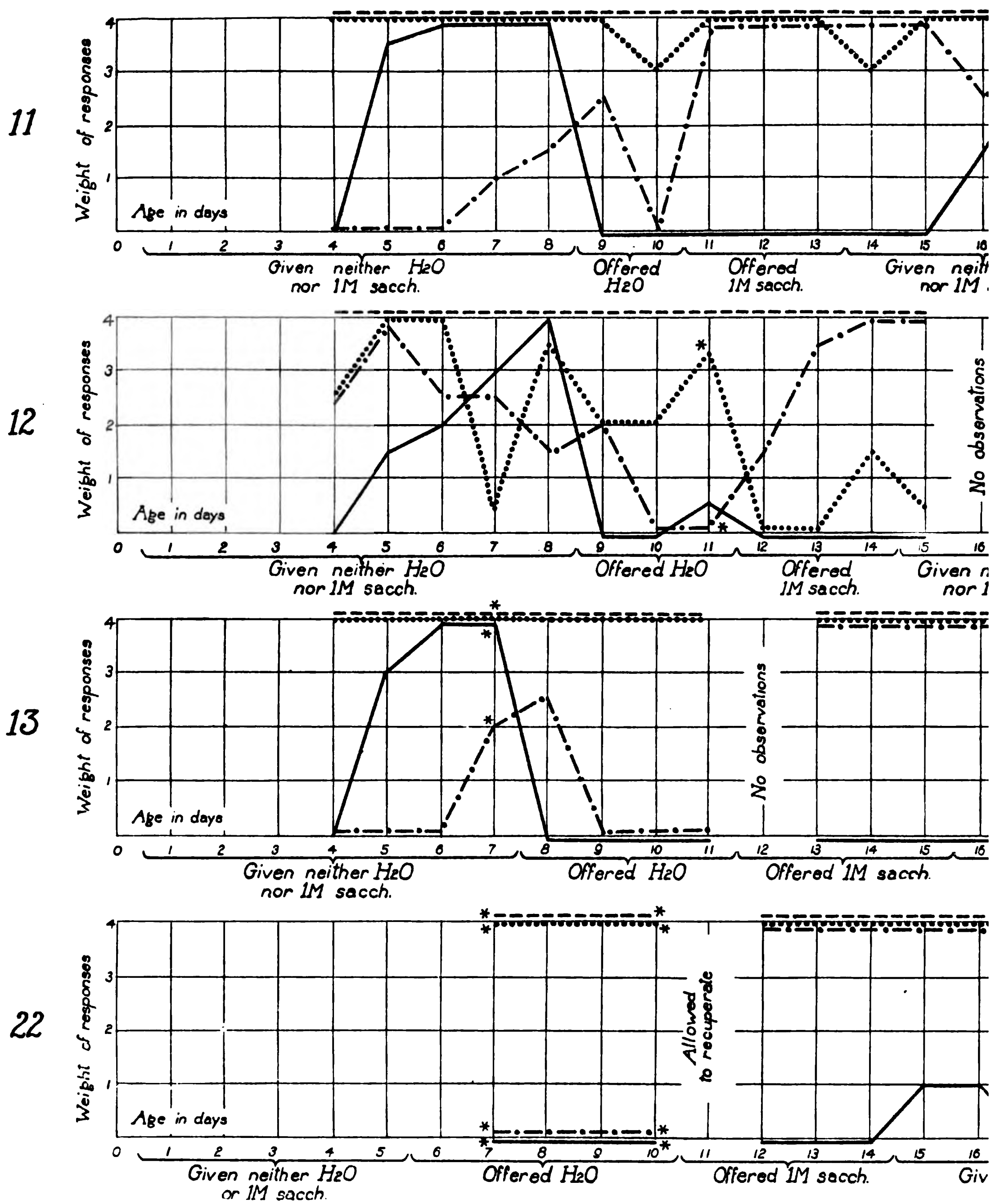
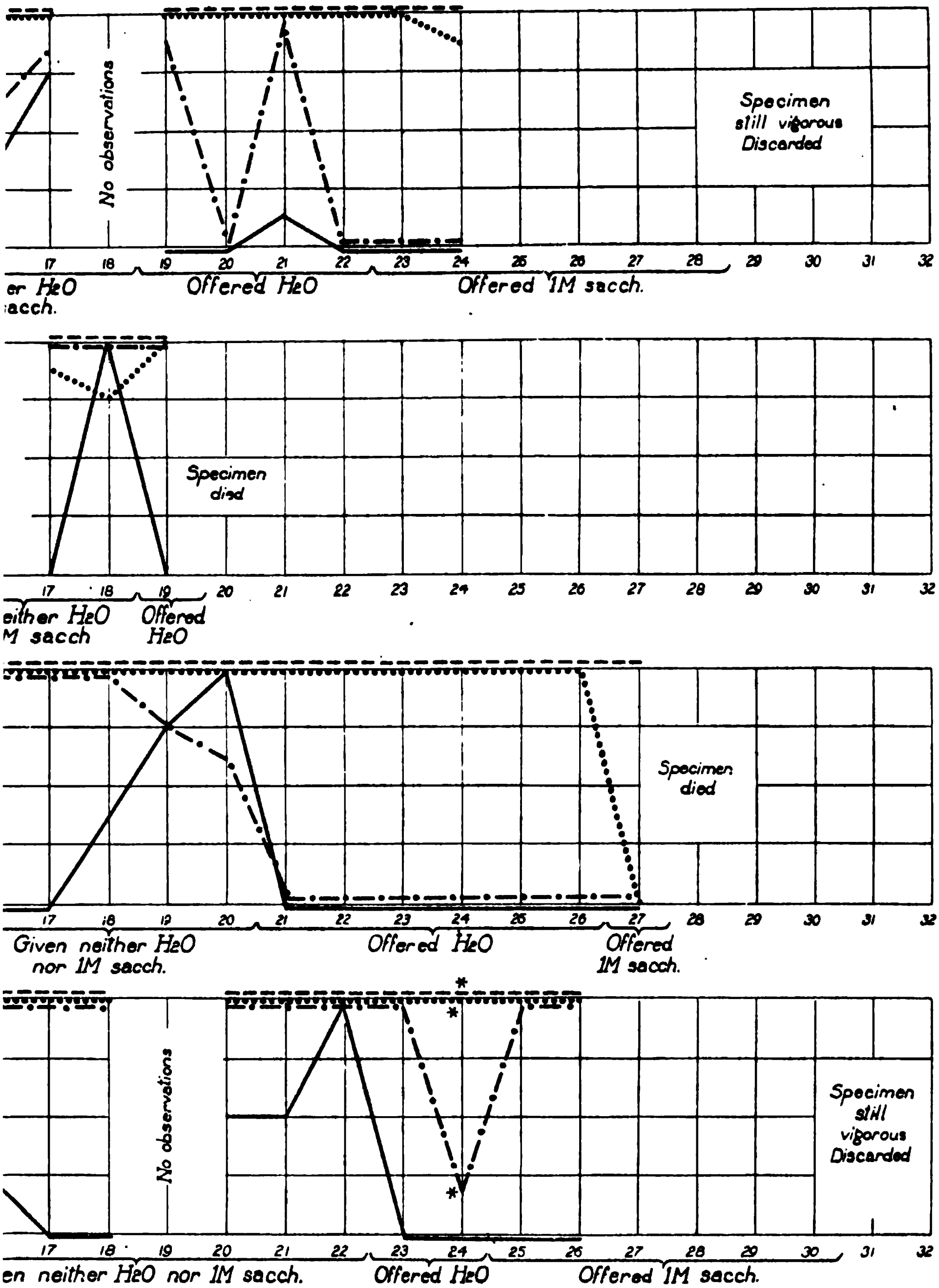


Fig. 3 Curves of response to various solutions and distilled water. ---------- 1M saccharos
Except where indicated by asterisk, the ordinate length represents the total weighted respons
asterisks indicate cases where for some reason, usually the weakness of the animal, it w
represents the per cent of response based on the number of trials actually made.



e; ....M/10 quinine hydrochloride; - - - - -2M sodium chloride; ——— distilled water.
e, as defined on page 59, obtained from four trials made in the course of a single day. The
impossible to complete the four trials of the day. In these instances the ordinate length

did the same excepting the last day of the experiment, when its responses suddenly dropped to 0. The total weighted response of all four specimens to the quinine solution was 84.7 per cent, as opposed to 100 per cent for 1M saccharose and 51.6 per cent for 2M NaCl. The amount of response produced by quinine was, therefore, intermediate between the other two substances.

A comparison of the curves for the quinine solution with those for sodium chloride (fig. 3) shows in every animal that there was a great diversity in the two responses. At times, the amount of response to each stimulus was the same; at other times, it was totally different. Here again, therefore, we must conclude that the two stimuli were differentiated.

A comparison of the saccharose and quinine curves yields no such conclusive evidence as the case above. For, while the two curves exhibit a rather wide divergence in animal no. 12, they very closely approximate one another in the other three animals. In this instance, however, there were distinctive differences of another sort. The rapidity with which the proboscis was extended to 1M saccharose has already been pointed out. As a comparison of the figures in table 2 will show, the response to quinine was very much slower. Thus, in animal no. 11 the average time required by quinine was seven to eight times that required by saccharose; in no. 12, thirteen to fourteen times; in no. 13, six to seven times, and in no. 22, three to four times. With 1M saccharose the extension of the proboscis began very shortly after the application of the stimulus and was rapidly completed. This was not the case with quinine. As a rule, the application of this stimulus was followed by a latent period lasting from a few seconds up to as many as sixty seconds, during which there was no sign of response. Then followed a period of reaction, beginning with slight relaxations of the proboscis which more or less gradually increased until extension was complete. The period of reaction also lasted from a few seconds up to fifteen or twenty seconds or even longer.[1]

[1] The long latent period together with the long period of extension necessitated the prolongation of a number of trials with the quinine solution from the usual duration of one minute to two minutes.

The average response to M/10 quinine hydrochloride thus differed strikingly from that to 1M saccharose. That this difference, together with the slight differences of distribution noted, indicates a discrimination of the two stimuli, seems to me not only possible, but very probable. A final statement, however, cannot be made with certainty.

From the evidence presented, it is quite clear that the tarsal organs of Pyrameis are chemoreceptors of a rather wide range of sensitivity. Through them the butterfly is able to differentiate such solutions as 1M saccharose and 2M NaCl from distilled water and from one another. It is also able to differentiate M/10 quinine hydrochloride from distilled water, from 2M NaCl, and probably from 1M saccharose. The appropriate stimulation of these organs leads to an extension of the proboscis, the initial act in food taking. The tarsal organs are thus organs of chemical sense, concerned in the discrimination of food substances, and may be properly considered as organs of taste.

GENERAL SUMMARY AND CONCLUSIONS

1. In Pyrameis atalanta Linn. each of the four ambulatory tarsi possesses contact chemoreceptors.

2. The appropriate stimulation of these receptors produces a response in the form of an extension of the proboscis.

3. The manifestation of this response varies somewhat, depending upon the chemical nature of the stimulus and the nutritional condition of the animal.

4. An intensive study of these differences of response shows that Pyrameis is able to distinguish the following substances from one another through its tarsal organs: distilled water, 1M saccharose, and 2M NaCl. It is also able to distinguish M/10 quinine hydrochloride from distilled water, from 2M NaCl, and probably from 1M saccharose.

5. The efficiency of distilled water in evoking the proboscis response is directly dependent upon the nutritional condition of the animal.

6. The responsiveness to 1M saccharose, 2M NaCl, and M/10 quinine hydrochloride shows no apparent relationship to the nutritional condition of the animal.

7. According to the scheme of measurement employed in the present paper, the total response of all animals together was 100 per cent to 1M saccharose, 84.7 per cent to M/10 quinine hydrochloride, and 51.6 per cent to 2M NaCl.

8. Since the organs of the tarsi are contact chemoreceptors, and since they are concerned with the discrimination of substances to be taken as food, they may be appropriately termed organs of taste.

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Resumen por la autora, Ann H. Morgan.

El sentido de la temperatura en la piel de la rana.

La piel de la rana contiene receptores bien definidos para el calor y el frío. Estos órganos presentan un intervalo de reacción comparativamente largo. Los receptores para el calor son estimulados por una temperatura de 39 a 43°C.; los receptores para el frío por una temperatura de 10°C. Esta respuesta es inmediata y aumenta en vigor a medida que crece el frio. La respuesta típica en el caso del frío es la rigidez y tensión de los músculos, pero puede haber una contracción hacia arriba semejante a la producida en la respuesta a la acción del calor. Las respuestas al calor y al frio pueden separarse una de otra y también de los sentidos tactil y químico y de los de contacto y dolor.

Translation by José F. Nonidez
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THE TEMPERATURE SENSES IN THE FROG'S SKIN

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ONE FIGURE

CONTENTS

INTRODUCTION

Is a temperature sense present in the frog's skin? Can it be isolated from the chemical and tactile senses which have already been shown to be there? Is it separable into the elementary senses of heat and cold as in the human skin? If so, what is the limitation and the nature of the responses to heat and cold?

It is in common knowledge that frogs go down into the mud in the winter and come up in the spring and an expectation of March and April that they will be heard on the warm nights. Their thermic susceptibility has long been known, both in nature and in the laboratory. Brown-Séquard ('47) alluded to the possible effect of temperature upon his reflex frogs which lived longest during the months from June to September, and Kunde

('60) recorded that reflex frogs dosed with strychnia were seized with spasms in a warm room, but became quiet when placed upon ice. A series of investigations followed these early suggestions, actual experiments on the direct and indirect effects of temperature upon the central nervous system (Tarchanow, '71; Archangelsky, '73; Freusberg, '75; Wundt, '76), the sensory nerve endings (Heinzmann, '72; Foster, '73; Rosenthal, '75; Sedgwick, '82), and the general behavior of the frog under stimulation by heat and cold. Recent workers upon its responses to light, electricity, sound, chemicals, and temperature have either shown something of its thermic sensitiveness (Korányi, '92; Parker, '03; Torelle, '03; Yerkes, '06; Pearse, '10) or have suggested the presence of a temperature sense in the skin. But its exclusive presence there, its existence as a separate sense, and the nature of its responses have not been adequately shown. It was with the hope of doing this and of answering the questions already suggested that the present study was undertaken.

The problem was suggested to me by Prof. G. H. Parker, and it gives me pleasure to express my appreciation of his friendly criticism and constant help.

HISTORICAL

Temperature studies upon the frog have covered a wide range of attack. Information regarding the effect of temperature upon the skin has come into the literature indirectly, usually in connection with special studies of a system of organs or the behavior of the whole organism. In the hope of showing these different aspects with greater clearness, I have discussed them by topics rather than in historical sequence.

Frogs respond to variations in temperature by visible motor reactions. This was established experimentally by Kunde ('60), Cayrade ('64), Goltz ('69), Tarchanow ('71, '72), Archangelsky ('73), Rosenthal ('75), Freusberg ('75), and Wundt ('76). The means of stimulation were partial or complete immersion in warm or cold water, dipping in warm or cool dilute acid, and ice packs and hot sand baths. From treatment of these kinds one group of workers (Kunde, '60; Richardson, '67; Weir-Mitchell, '67;

Rosenthal, '75) maintained that cold caused a depression in reflex excitability, except in the case of ice packs, which increased it (Richardson, '67; Weir-Mitchell, '67; Wundt, '67), and another group were of the opinion that heat properly applied also caused excess excitability (Cayrade, '64; Goltz, '69; Tarchanow, '71, '72; Freusberg, '75).

When cold or heat are applied very gradually to a frog the reactions decrease in extent and vigor. There has been a good deal of disagreement in the literature, regarding reactions to gradually applied stimuli. The question was opened by Goltz ('69), who immersed normal and reflex frogs in water of gradually increasing temperatures. When slowly stimulated up to 30°C., the normal frog became violent, but the reflex frog remained inert. Goltz's main purpose had been to show the difference between the two conditions in the animal, and he immediately declared the lassitude of the reflex frog due to its brainless state. By the same method, Tarchanow ('71, '72) secured similar results on normal frogs. The next year Heinzmann ('72) continued similar experiments from the point of view that the sensory nerves might be affected by a stimulus increasing in intensity so slowly that destruction of the nerve would result before a reaction could occur. Normal and reflex frogs were heated with the expected results to both of them, and these were explained as due to the very gradual succession of the stimuli. In 1875 Fratscher repeated these experiments with identical results. The quiet normal frogs of Heinzmann and Fratscher were thus pitted against the violent normal frogs of Goltz and Tarchanow, but the main conclusion seems to have been that no reaction would result if stimulation were applied with sufficient gradualness.

Foster ('73) had previously questioned Goltz's statement that brainless frogs would give no reaction to stimuli to which normal frogs reacted so vigorously. He immersed reflex frogs 'locally and totally' and obtained very different results in the two cases. When large areas of the body were immersed there was no response, but when only the toes were dipped, no matter how gradually the heat was increased, they were always withdrawn at about 35°C. This peculiar result was explained by Foster

on the ground that immersion of the larger areas heated the blood, which in turn warmed the spinal cord and reduced its irritability. With the stimulation of the small area no such general warming could take place, and hence the normal irritability of the cord was retained and the vigorous response followed.

Certain puzzling phases immediately presented themselves, and Sedgwick ('82) repeated the experiments upon which this explanation was based. He suspended the reflex frogs in the manner described by Foster and at once discovered that in this upright position the heart was practically empty and could not possibly circulate the blood as stated by Foster.

From this tangle of statements the best evidence seems to show that the reflex frog will respond to heat at certain degrees, no matter how gradually it is applied, but that the extent and vigor of these responses may be reduced by the graded application of the stimulus.

Effect of heating and cooling the spinal cord. With the object of stimulating the spinal cord, Archangelsky ('73) suspended reflex frogs with their trunks surrounded by a jacket of hot air which produced a rise of excitability, and by a jacket of slowly heated air which produced no change. Tarchanow ('71, '72) stimulated the cord directly with an ice pack, thereby causing a depression of reflexes.

Frogs can withstand a temperature as low as 6°C. The body temperature of frogs was recorded by Milne-Edwards ('68) and by Rogers and Lewis in 1916. Knauthe ('91) and Müller-Erzback ('91) froze frogs in water and exposed them to temperatures of −4°C. to −6°C. for several hours. Maurel et Lagriffe ('00) studied the effect of temperatures from −4°C. to 41°C. and maintained that a frog may survive a temperature of 0°C. or even −3°C.

Respiration is quickened under stimulation by heat. When Babák ('13) warmed the skin of a reflex frog, the speed of respiratory movements was quickened, and when he cooled it, correspondingly the speed was decreased.

Frogs which are immersed in cold water will swim downward and will remain at the bottom a greater percentage of the time as the cold

is increased. Frogs which Torelle ('03) placed in water of 10°C. immediately swam down and remained below, usually with legs stiffly outstretched. In 1918 Brooks corroborated this by a series of detailed observations on frogs which were placed in water of decreasing temperature. As the water was cooled the frogs remained for a shorter and shorter time at the surface till at 5°C. they settled to the bottom and remained there.

The skin is sensitive to variations in the temperature of air, of water, and of acid solutions. Comparing the sensibility of the skin and afferent nerves by treatment with warm and cool acid solutions, Tarchanow ('72) was the first to point out that the thermal end-organs must be in the skin and that the quicker response to the warmer acid solutions was due to an increased irritability in the nerve endings, agreeing in this with Archangelsky ('73) who had used the same stimulus. This sensitiveness of the skin has been mentioned or investigated by recent workers in connection with studies of other sense organs, and Korányi ('93) and Pearse ('10) found the frog's integument responsive to both light and heat. Pearse secured responses from frogs whose feet were dipped in water at 40°C. and 45°C. and Reese ('06) obtained similar results from Cryptobranchus, while Parker ('03) and Yerkes ('06) both alluded to the susceptibility of the skin to changes of temperature.

Warmth produces a positive and cold a negative response to light. When frogs were placed in warm air or water they moved toward the light, but in the same media at 8°C. they moved away from the light (Torelle, '03). L. J. Cole ('07) secured similar results when he placed a frog in a dark box between a large and a small illuminated area at the opposite ends. When the frog was cooled to 6°C. and 10°C. it would move toward the smaller area, but when warmed it would immediately move toward the larger one. In order to compare the relative effects of light and heat, Pearse ('10) arranged a series of tubes, with a measured heat radiation upon the sides of a totally dark box. Another box contained a light whose heat output was one-half that of the pipes. Eyeless toads placed in these boxes proved to be almost totally indifferent to the heat, but were strongly phototropic, showing that light

and heat were unlike in effect and that the photoreceptors were much more easily excited than the receptors for heat. A slight difference in light, on the other hand, made no impression on frogs with which Torelle ('03) worked. They swam up and down in the jars regardless of adjustments of light and dark.

Frogs are stereotropic in temperatures between 10°C. and 4°C. When Torelle placed frogs in water cooled to 10°C. or below, they flattened their bodies against the bottom or crept under rocks placed on the floor of the aquarium.

Effect of temperature on responses to electricity. An electric current which produced tetanic movements on a warm frog showed retardation when the frog was cooled (Kunde, '60).

METHODS

The experiments which follow were performed upon green frogs (Rana clamitans) and leopard frogs (Rana pipiens) in a laboratory the temperature of which varied between 18°C. and 23°C. The work was done between October and January upon animals which were kept in a basement tank and brought into the laboratory at least two days before they were used for experimentation.

For all except one experiment, the front part of the head was removed by a single transverse cut made just in front of the eardrums. Through the lower jaw thus left intact a loop of silk was drawn, and by this the frog was suspended, thus avoiding the irritation caused by the repeated use of a metal hook.

Frogs were hung from an extension bar, attached to a standard; the bar could be easily raised and lowered. They were completely immersed in a bath of water at the beginning of each experiment, and at certain intervals during treatment in order to keep the temperature normal, the skin moist, and free from particles of dust. At the beginning of an experiment the temperature of the room, bath water and frog were taken, the latter being secured by putting a thermometer through the mouth and down into the stomach. Records of these temperatures have been given with each experiment recorded in this paper. The experimental frogs were easily kept in good condition and usually lived from four to five weeks.

The surface of the foot was the only area treated. Sometimes one foot was stimulated and the other kept as a check, but in most cases there was an alternate stimulation of the normal feet, or of the normal and the treated foot. Baths of water and applications of stimuli were given at definite intervals which were kept uniform through each experiment. Preliminary experiments were made with each different kind of stimulation in order to find out what reaction might be expected.

At each test a definite allowance of time was given, and if the reaction did not occur within that period the stimulus was regarded as producing no reaction and recorded as ∞. In the tables the period just described is termed the reaction allowance. The interval which actually elapsed between the application of the stimulus and the reaction was taken in seconds with a stop-watch and recorded with the description of the response. No periods less than half a second were recorded. Intervals which separated stimulations sufficiently to prevent exhaustion were also selected by experiment. These have been designated the stimulation intervals.

During the experiments the normal feet were kept in 'bath water' unless actually undergoing stimulation. In experiments made under cocaine treatment the foot was always returned to the cocaine solution after it had been immersed in the stimulant. In the preliminary part of this work a good deal of trouble was experienced by the washing out of the cocaine, so this procedure was found necessary. A solution of 1 per cent cocaine was the only anaesthetic used.

OBSERVATIONS

Responses to heat

The first experiments of this series were made in order to determine whether the frog's foot would regularly react to heat, and if so to what degree of heat. A typical heat response was also looked for, a position or movement which should recur in many different individuals. Both feet of the experimental frog were kept in normal condition. In the first experiments considered

(table 1) only the right foot was stimulated and the left served as a check. The right foot was first immersed in water at 30°C. and at intervals of two minutes after that in baths increasing each time by 1°C. from 30°C. to 50°C.: no responses occurred below 39°C., and in some instances none below 43°C. As the heat was increased the vigor of the response was also increased

TABLE 1

Reaction intervals in seconds of frogs' right feet subjected to temperatures ranging at one degree intervals from 30° to 50°C. No reactions (indicated by ∞) were obtained at temperatures of 38° or lower, hence this part of the table is condensed. Feet normal. Reaction allowance, 30 seconds. Stimulation interval, 2 minutes

Number of individual	2	4	6	7	13	8	9	10	11	11	3
Number of experiment	1	1	1	1	1	1A	1A	1	3	4	1
Temperature of room	24°	19°	21°	24°	22°	21°	25°	22°	21°	24°	19°
Temperature of bath water	21°	17°	18°	21°	18°	20°	20°	19°	18°	21°	17°
Temperature of frog	22°	18°	19°	22°	19°	20°	23°	20°	19°	21°	18°
Stimulated by water 30°C.											
to 38°C	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
39°C	∞	∞	∞	∞	∞	∞	∞	∞	∞	10	∞
40°C	∞	24	∞	∞	∞	∞	∞	15	17	5	12
41°C	9	14	∞	22	∞	29	∞	22	8	6	17
42°C	9	11	∞	∞	∞	14	∞	15	10	4	13
43°C	6	10	12	15	5	8	21	3	7	3	12
44°C	4	6	9	12	5	7	20	4	6	2	8
45°C	3	4	5	9	7	8	12	5	4	4	4
46°C	2	3	4	8	3	5	13	5	4	2	7
47°C	2	4	3	5	3	6	8	4	3	2	4
48°C	2	3	2	3	2	2	6	4	3	2	3
49°C	2	1	2	4	1	3	5	3	2	2	3
50°C	2	1	1	2	1	3	5	2	3	2	2

and the reaction interval became shorter and shorter. The heat response was a vigorous upward jerk of the foot, so uniform that usually no attempt to describe it has been made except by the word 'jerk' and the statement of the length of the reaction interval, i.e., the time between the application of the stimulus and the reaction itself.

The next step was to discover whether there would be different results if the heat was applied with differences of temperature

greater than 1°. It will be remembered that some of the early workers (Goltz, '69; Tarchanow, '71, '72) maintained that if heat were increased slowly enough a frog might be subjected to an extreme degree without making responding resistance. No such results were secured in this investigation even when the heat was increased very slowly. A frog's foot was placed in a beaker of water at 2°C., and a stream of warm water allowed to flow into this which brought it up to 45°C. with almost imperceptible slowness. Although care was taken to prevent the foot from

TABLE 2

Responses in seconds to heat increasing by 5°C. at each stage of stimulation. Feet normal. Reaction allowance, 30 seconds. Stimulation interval, 2 minutes for nos. 8, 9, 10, and 5 minutes for no. 11. ∞ = no reaction

Number of individual	8	8	8	8	10	10	9	9	11	11	11	11
Number of experiment	6A	6A	6A	6A	3	3	1B	1B	5	5	5a	5a
Foot stimulated	R	L	R	L	R	L	R	L	R	L	R	L
Temperature of room	24°	24°	24°	24°	20°	20°	25°	25°	22°	22°	25°	25°
Temperature of bath water	20°	20°	21°	21°	18°	18°	20°	20°	22°	22°	20°	20°
Temperature of frog	23°	23°	22°	22°	18°	18°	23°	23°	22°	22°	23°	23°
Stimulated by water at												
30°C	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
35°C	∞	∞	∞	15	∞	∞	∞	∞	25	29	11	12
40°C	7	22	4	5	19	14	12	21	7	12	4	4
45°C	3	3	1	2	6	18	5	12	6	8	1	1
50°C	2	2	1	1	3	4	4	3				

being affected by this stream, the feet were invariably lifted before the heat had reached 45°C.

Variations in the stimulation time and in the heat increments were also tried (table 2). Heat was increased by 5°C. at each stimulation and the right and left feet of the frog were dipped at one-, two-, and five-minute intervals. Of nine frogs used only four reacted at a degree lower than that to which the frogs responded which were subjected to 1°C. increases. The results showed the tendency toward the later reaction with gradually applied stimuli, but also suggested that individual idiosyncrasies

were a factor and that certain frogs were especially sensitive to heat. This opinion was further justified by a series of tests repeated over and over on particular individuals. The right foot of each of these frogs was stimulated by hot water whose temperature ranged from 25°C. to 50°C. and at intervals of 5°, the experiment being repeated seven times consecutively (table 3). This was done to find out whether each frog would preserve its individual eccentricities toward heat in successive tests and

TABLE 3

Responses in seconds of two frogs to hot water whose temperature was increased by 5°C. at each stimulation. Each series repeated consecutively seven times. Feet normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no reaction

Number of individual	11							11							14						
Number of experiment	1							2							1						
Foot stimulated	R							R							R						
Temperature of room	22°							22°							20°						
Temperature of bath water	20°							20°							18°						
Temperature of frog	22°							22°							21°						
Stimulated by water at																					
25°C.	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
30°C.	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
35°C.	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	6	7	4	∞	4	3	12
40°C.	∞	9	10	5	5	7	5	5	22	∞	19	6	15	∞	3	5	4	3	4	2	3
45°C.	3	3	3	3	3	3	2	3	6	2	4	3	3	5	2	3	2	3	2	1	2
50°C.	2	1	1	1	2	1	1	2	3	3	2	2	2	2	1	2	1	2	2	1	1

on different days. Two records selected (no. 11, 1; no. 11, 2) were of a frog of average susceptibility and the tests 1 and 2 were made on different days, and the third (no. 14, 1) was chosen because it showed unusual sensitiveness. Both types were fairly common and the sensitiveness or dullness persisted with repeated stimulations in nearly all the animals tested. These results indicated that the speed of applying stimuli is not the only factor present in quick or slow responses.

The heat responses were characteristically sharp upward jerks of the leg. As it was pulled upward the toes were held together

with the web folded between them, a very different position from that later observed in the response to cold. Responses increased in vigor with the increase in heat and were often followed by jerking and twisting of the whole body.

The interval between stimulation and response to heat (actual reaction time) was relatively long. Frogs which responded unfailingly at 40°C. might not do so for twenty seconds after contact with the stimulant. This also contrasted with the short reaction time of the cold response and indicated that the receptors for warmth in the frog's skin lie deep, and those for cold are superficial as in the human skin. The reaction time showed a fairly regular decrease with the increase of the heat. This was observed in all experiments with increasing temperatures. Thus far the experiments with heat show a characteristic response which occurred with clock-like regularity; with increasing heat the first responses were between 35°C. and 43°C. and the reaction time was comparatively long, but decreased as the heat increased. The range of the first type of response was only slightly modified when the stimulus was very gradually applied.

Is there a temperature sense in the skin? With regular responses thus secured in the foot, the next problem was to show that these responses were not due to stimulus of the muscles, and to prove the exclusive presence of the temperature sense in the skin.

The skin was desensitized by three different methods. A series of tests paralleling the preceding was made upon frogs with one foot normal and the other treated with a solution of 1 per cent cocaine, upon frogs with one foot normal and the other with the sciatic nerve cut, and upon frogs with one leg normal and the skin removed to the ankle from the other.

The cocaine method of desensitizing the skin has been used by L. W. Cole ('10), Crozier ('16), and others. By varying the length of treatment with the 1 per cent solution of cocaine, different senses in the skin can be affected or even eliminated. In experiments which involved only the heat sense the foot was cocained for thirty minutes immediately preceding the experiment. It was also immersed in the cocaine solution during the

intervals between stimulations. Cocained and normal feet were alternately stimulated by water increasing in heat by 1° at a time from 30°C. to 50°C. (table 4). The first reaction of the cocained foot was at 45°C. after a reaction interval of 24 seconds, but the first response of the normal one was at 40°C. after a reaction interval of 5 seconds. The average degree of first re-

TABLE 4

Responses in seconds to heat increasing by 1°C. at each stimulation. Right foot cocained 30 minutes. Left foot normal. Reaction allowance 30 seconds. Stimulation time, 2 minutes. ∞ = no response

Number of individual	11	11	48	48	47	47	46	46
Number of experiment	8	8	7	7	8	8	5	5
Foot stimulated	R	L	R	L	R	L	R	L
Temperature of room	16°	16°	19°	19°	22°	22°	21°	22°
Temperature of bath water	15°	15°	17°	17°	19°	19°	18°	18°
Temperature of frog	15°	15°	18°	18°	20°	20°	19°	19°
Stimulated by water at 38°C	∞	∞	∞	∞	∞	∞	∞	∞
39°C	∞	∞	∞	∞	∞	∞	∞	∞
40°C	∞	5	∞	4	∞	5	∞	4
41°C	∞	5	∞	6	∞	6	∞	3
42°C	∞	4	∞	5	∞	4	∞	4
43°C	∞	1	∞	4	∞	3	∞	5
44°C	∞	4	∞	3	∞	4	∞	4
45°C	24	2	∞	4	20	3	18	3
46°C	24	2	25	3	21	3	17	2
47°C	24	1	23	2	18	2	20	2
48°C	28	1	22	2	24	1	22	2
49°C	26	1	25	1	24	1	24	1
50°C	30	1	28	1	25	1	25	1

sponse for the cocained foot was 45°C. after an average reaction time of 21.75 seconds. For the normal foot the average first response was at 40°C. after an average reaction interval of 4.5 seconds (table 4).

Following this twelve more frogs were tested by similar methods, but with the stimulations beginning at 15°C. and continuing by 5°C. intervals to 45°C. (table 5). Since no responses were secured, the lower degrees 15°C. to 30°C. have not been

included in the table. At 40°C. the normal foot of each frog responded after an average reaction time of 10 seconds. At 45°C. the cocained foot responded after an average reaction interval of 16.75 seconds against an average of 8 seconds for the normal foot. Such results showed that when the skin was anaesthetized the temperature sense was affected—a clear proof that the heat receptor is located only in the skin.

In order to test this question further, however, the cutaneous nerve supply was cut off by severing the sciatic nerve at the thigh. In four frogs thus treated the injured and the normal leg were

TABLE 5

Responses in seconds to heat increasing by 5°C. at each stimulation in a range of 15°C. to 45°C. Right foot cocained 30 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.				REACTIONS	
		Room	Bath water	Frog	Stimulus	Right foot	Left foot
25	2	16°	16°	15°	35°	∞	∞
25	2	16°	16°	15°	40	∞	10
25	2	16°	16°	15°	45	25	10
27	2	19°	15°	16°	35	∞	∞
27	2	19°	15°	16°	40	∞	5
27	2	19°	15°	16°	45	10	2
30	1	18°	15°	16°	35	∞	∞
30	1	18°	15°	16°	40	∞	15
30	1	18°	15°	16°	45	15	12

alternately stimulated by the same degree of heat, but not the slightest response was obtained from the denervated leg, showing that the response was in no sense purely muscular. From three other frogs the skin of one foot was removed. The feet of one were alternately immersed in water of increasing temperature, those of the other two were dipped in water at 45°C. Again there was no reaction except in the normal foot whose responses were uniform with those already secured. Hence the skin of the frog's foot is essential to these reactions. The results of these experiments show that the temperature sense is at once affected by cocaine, and that it is entirely eliminated by destruction of the cutaneous nerve supply or by removal of the skin.

Independence of the responses to touch and heat. In the human skin responses to tactile stimulations occur quickly, but pain and heat have a longer reaction interval. In the frog reaction times to touch and heat were first compared in the normal skin as a step toward the separation of the two senses and finally from those of cold, pain, and the chemical senses.

In these comparative experiments the heat stimulation was conducted as before. In applying the tactile stimulus a simple

TABLE 6

Responses in seconds to heat at 40°C. and to heat increasing by 5°C. at each stimulation in a range of 25°C. to 50°C. Left foot with skin removed. Right foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no response

Number of individual	8	8	8	8	9	9		13	13
Number of experiment	4	4	5	5	4	4		4	4
Foot stimulated	R	L	R	L	R	L		R	L
Temperature of room	22°	22°	22°	22°	24°	24°		21°	21°
Temperature of bath water	18°	18°	18°	18°	18°	18°		18°	18°
Temperature of frog	17°	17°	17°	17°	18°	18°		19°	19°
Stimulated by water at 40°C	3	∞	10	∞	8	∞	25°C.	∞	∞
40°C	6	∞	12	∞	8	∞	30°	∞	∞
40°C	3	∞	8	∞	8	∞	35°	20	∞
40°C	3	∞	6	∞	5	∞	40°	10	∞
40°C	3	∞	15	∞	6	∞	45°	4	∞
40°C	3	∞	10	∞	6	∞	50°	3	∞
40°C	3	∞	7	∞	7	∞			
40°C	3	∞	5	∞	5	∞			

device was employed by which the stimulus could be kept uniform and brought to bear on a particular area (fig. 1). This device consisted of a single iron standard from the top of which the frog was suspended by an adjustable arm. Below this were two adjustable arms also provided with clamps. Into the lower and longer one a small board was fastened by a flexible wire so that the board could be easily turned in any direction. This board served to support the frog's foot very lightly and when flooded with water there was probably little tactile stimulation from it.

The upper clamp held a large tube through which common steel shot could be easily rolled. This tube was adjustable in the clamp so that its angle of inclination could be easily changed. When a tactile stimulation was to be made the frog was suspended

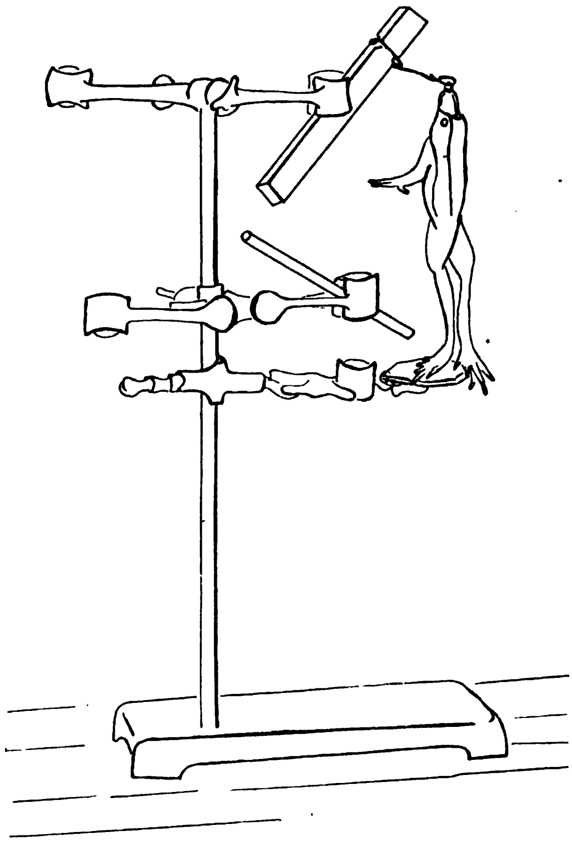

Fig. 1 Apparatus for suspending frog and for tactile stimulation by falling shot.

with its foot resting very lightly on the support, and with the glass tube trained upon the spot to be stimulated, so that when the shot was rolled through the tube it would strike the proper surface of the foot. The distance between the surface of the foot and the end of the tube, the inclination of the tube at 45°,

and the size of the shot were kept constant, but the length of the tube was varied according to the strength of the stimulus desired. The length of the tube and one or two words descriptive of the reaction have been recorded in each table. The results secured

TABLE 7

Responses in seconds to touch and heat. Shot rolled through tube striking upon the surface of the foot. Feet normal. Reaction allowance 30 seconds. Stimulation time, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY		REACTIONS	
		Room	Bath water	Frog	Shot; length of tube used for shot	Water	Right foot	Left foot
					inches			
12	6	20°	19°	20°	2		1 lifted	1 lifted
12	6	20°	19°	20°	2		1 lifted	1 lifted
12	6	20°	19°	20°	2		1 lifted	1 lifted
12	6	20°	19°	20°		45°C.	6 jerked	3 jerked
13	5	16°	16°	15°	2		1 lifted	1 lifted
13	5	16°	16°	15°	2		1 lifted	∞
13	5	16°	16°	15°	2		1 lifted	∞
13	5	16°	16°	15°		45°C.	6 lifted	3 lifted
15	3	19°	11°	11°	2		1 jerked	1 jerked
15	3	19°	11°	11°	2		1 jerked	1 jerked
15	3	19°	11°	11°	2		∞	1 lifted
15	3	19°	11°	11°		45°C.	6 jerked	5 jerked
16	1	23°	19°	18°	2		∞	∞
16	1	23°	19°	18°	2		1 jerked	1 jerked
16	1	23°	19°	18°	2		1 lifted	1 jerked
16	1	23°	19°	18°	2		1 lifted	1 lifted
16	1	23°	19°	18°		45°C.	14 lifted	9 lifted
13	3	22°	19°	18°	2		1 jerked	1 lifted
13	3	22°	19°	18°	2		1 jerked	1 lifted
13	3	22°	19°	18°	2		∞	∞
13	3	22°	19°	18°		45°C.	5 jerked	5 jerked

by stimulating the normal feet by the stroke of the shot, also by heat, are shown in table 7. Twelve experiments were made and the records of five were selected because these showed the early exhaustion of the tactile sense and the persistent presence of the heat response, the shorter reaction time and gentler response to touch contrasted against the abrupt reaction of heat following a long reaction time.

If the receptors for heat lie deeper than those for touch and cold, as is true in the human skin, it seemed that it would be possible to affect them sooner by a solution of cocaine and thus demonstrate the independence of the two senses. This was attempted by stimulating a normal and a cocained foot with the falling shot and with heat. Some difficulty was experienced in keeping the skin from being exhausted by tactile stimulation before the cocaine treatment was finished. Frogs which struggled had to be repeatedly adjusted, and this could hardly be accomplished without touching the frog somewhere. It was necessary, therefore, to perform a good many experiments and to select quiet frogs. Fifteen such frogs were used, and from their records the five in table 8 were selected. In these fifteen frogs

TABLE 8

Responses in seconds to touch and heat. Touch stimulation shot, rolled through tube striking upon surface of the foot. Right foot cocained 10 to 20 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = *no response*

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY WATER	STIMULATED BY SHOT; LENGTH OF TUBE	COCAINE TREATMENT	REACTIONS	
		Room	Bath water	Frog				Right foot	Left foot
						inches	*minutes*		
13	4	22°	18°	19°		2	20	∞	1 lifted
13	4	22°	18°	19°		2	20	∞	1 jerked
13	4	22°	18°	19°	45°C.		20	11 jerked	1 jerked
15	4	18°	19°	17°		2	20	∞	1 lifted
15	4	18°	19°	17°		2	20	∞	1 lifted
15	4	18°	19°	17°	45°C.		20	8 jerked	4 lifted
15	7	19°	19°	18°		5	15	∞	1 lifted
15	7	19°	19°	18°		5	15	∞	1 lifted
15	7	19°	19°	18°	45°C.		15	8 jerked	4 lifted
13	6	16°	16°	16°		2	10	∞	1 lifted
13	6	16°	16°	16°		2	10	∞	1 contracted
13	6	16°	16°	16°	45°C.		10	8 jerked	4 jerked
12	7	23°	19°	20°		2	20	∞	1 lifted
12	7	23°	19°	20°		2	20	∞	1 lifted
12	7	23°	19°	20°	45°C.		20	4 jerked	2 jerked

the normal foot never failed to react to touch except in two cases, clearly caused by a faulty technique. The cocained foot failed to react to the strike of the falling shot at any time (table 8), but both the normal and cocained foot reacted regularly and with the same retardation which had been affected by the cocaine in previous experiments (tables 4, 5). The conclusion is that independent heat receptors are present in the foot of the frog and that a complete separation of the touch and the heat sense had been affected.

TABLE 9

Responses in seconds to pain and heat. Pain stimulation by pricking outer side of fifth toe. Feet normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY NEEDLE	STIMULATED BY WATER	REACTIONS	
		Room	Bath water	Frog			Right foot	Left foot
10	10	20°	19°	18°	Pricking		1 jerked	1 jerked
10	10	20°	19°	18°	Pricking		1 jerked	1 jerked
10	10	20°	19°	18°		40°C.	6 jerked	7 jerked
50	1	21°	20°	19°	Pricking		1 jerked	1 jerked
50	1	21°	20°	19°	Pricking		1 jerked	1 jerked
50	1	21°	20°	19°		40°C.	6 jerked	7 jerked

Independence of the responses to pain and heat. The method used in this separation was dipping the foot in water at 40°C. and pricking the skin on the outer side of the fifth toe. No degree of heat higher than 40°C. was used, because of the possibility that the higher degrees of heat might be painful and the two responses thus confused. Pricking the web between the third and fourth toes was first tried, the particular web being quite arbitrarily selected for stimulation. In some cases the foot would react to pricking done anywhere on this web, in other cases it would react to it in certain areas only, and in still other cases the foot would fail or almost fail to give any reaction at all to pricking anywhere on this web. Other webs were afterward tried with much the same result. When the skin on the

side of the toe was pricked, the normal foot never failed to react, though care was taken that the needle did not pass through underlying tissue.

TABLE 10

Responses in seconds to pain and heat. Pain stimulations by pricking the side of the fifth toe. Right foot cocained 50 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = *no response*

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY NEEDLE	STIMULATED BY WATER	REACTIONS	
		Room	Bath water	Frog			Right foot	Left foot
47	1	18°	16°	16°		40°C.	∞	1 jerked
47	1	18°	16°	16°	Pricking		1 jerked	1 jerked
47	1	18°	16°	16°		40°	∞	8 jerked
47	1	18°	16°	16°	Pricking		1 jerked	1 jerked
47	1	18°	16°	16°		40°	∞	15 jerked
48	1	21°	18°	16°		40°	∞	6 jerked
48	1	21°	18°	16°	Pricking		1 jerked	1 jerked
48	1	21°	18°	16°		40°	∞	5 jerked
48	1	21°	18°	16°	Pricking		1 jerked	1 jerked
48	1	21°	18°	16°		40°	∞	10 jerked
49	1	21°	17°	16°		40°	∞	9 jerked
49	1	21°	17°	16°	Pricking		1 jerked	1 jerked
49	1	21°	17°	16°		40°	28 jerked	12 jerked
49	1	21°	17°	16°	Pricking		1 jerked	1 jerked
49	1	21°	17°	16°		40°	29 lifted	12 jumped
47	2	20°	17°	16°		40°	∞	7 jerked
47	2	20°	17°	16°	Pricking		1 jerked	1 jerked
47	2	20°	17°	16°		40°	25 lifted	6 jerked
47	2	20°	17°	16°	Pricking		1 jerked	1 jerked
47	2	20°	17°	16°		40°	∞	15 jumped
47	3	16°	12°	12°		40°	∞	12 jerked
47	3	16°	12°	12°	Pricking		1 lifted	1 lifted
47	3	16°	12°	12°		40°	25 lifted	5 lifted
47	3	16°	12°	12°	Pricking		1 lifted	1 lifted
47	3	16°	12°	12°		40°	∞	25 lifted

This comparison of pain and heat was first tried upon the normal feet with a regular, sudden, and vigorous response to the pricking and the characteristic jerk and extended reaction time after heat stimulation. The response to heat was eliminated or greatly retarded by the fifty minutes' treatment with cocaine

given to the right foot, but the normal left foot showed either a normal reaction time or one slightly longer than usual. Pricking produced immediate and vigorous response in both the cocained and normal foot even after a long period.

Independence of the responses to acid solution and heat. The existence of the common chemical sense in the frog's skin was clearly shown by L. W. Cole ('10) and by Crozier ('16). Cole treated the skin with cocaine and found it sensitive to ammonium chloride after response to pain had wholly disappeared, and by

TABLE 11

Responses in seconds to acid solution and to heat. Acid stimulation by 0.5 per cent HCl solution. Right foot cocained 45 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation interval, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY WATER	STIMULATED BY HCl	REACTIONS	
		Room	Bath water	Frog			Right foot	Left foot
						per cent		
47	4	16°	12°	12°	40°		∞	25 lifted
47	4	16°	12°	12°		0.5	1 lifted	1 lifted
48	2	19°	16°	18°	40°		∞	12 lifted
48	2	19°	16°	18°		0.5		1 jerked
49	2	19°	15°	13°	40°		∞	25 lifted
49	2	19°	15°	13°		0.5	1 lifted	1 lifted
47	5	19°	15°	14°	40°		∞	5 lifted
47	5	19°	15°	14°		0.5	1 lifted	1 jerked
46	6	19°	15°	13°	40°		∞	7 lifted
46	6	19°	15°	13°		0.5	7 lifted	3 lifted

similar treatment Crozier secured responses to N/20 formic acid after response to pinching had altogether ceased.

In the separation of chemical and temperature senses the previous method of comparison was repeated. Water at 40°C. and a solution of one-half of 1 per cent hydrochloric acid were used as stimulants. After forty-five minutes of the cocaine treatment there was no response to heat by the right foot, although its reaction to the acid solution was as prompt and vigorous as in the left. These facts are quite in accord with those observed by Crozier.

Responses to cold

Normal frogs respond promptly and definitely when they are placed in water sufficiently cold. Such behavior was observed by both Torelle ('03) and Brooks ('18), who recorded that frogs immersed in water of 5°C. swam downward, attempted to regain the surface a couple of times, but finally sank to the bottom and remained there.

In studying the effect of cold, experiments such as had been made by Torelle and by Brooks were repeated in the reflex frogs so as to compare their behavior with that of normal frogs.

The temperature of the frog was taken before and after the experiment. Four gallons of water at a temperature of 4°C. to 5°C. was kept in an aquarium jar in the laboratory and frogs were placed in this water for periods of two minutes. Short records of observations were made by fifteen-second intervals (table 12). The first responses were uniformly either a spasmodic jump or a 'set' of the whole body like the 'freeze' of a rabbit, followed by a series of jumps, then a gradual sinking in which the body fell rigidly backward or forward and finally rested stiffly on the bottom of the jar with the legs extended. These responses were generally very uniform, but when there were individual peculiarities they were persistent. For example, frog no. 16 was tested on different days, but its immediate response to cold was always a sudden 'set' in which the legs were tightly flexed to the body, the toes extended, with the web tightly stretched and the soles out. In this position the frog would sink to the bottom without a contraction; it did not, however, lose its rigidity even when it was turned from side to side with a rod.

A few similar observations were made upon normal frogs to compare their behavior with reflex frogs. The two records presented in table 13 are typical of a half-dozen. Normal frogs floated longer at the surface than reflex frogs did, but in other respects the behavior of the two kinds was essentially the same.

Alternate dipping of the feet in water at 2°C. resulted in much the same response as did total immersion. The characteristic

TABLE 12

Responses of reflex frogs to total immersion in water at 4°C. to 5°C. Entire skin normal. Observation period of 2 minutes divided into 8 sections of 15 seconds each

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS
		Cold bath	Frog									
			Before bath	After bath								
10	10	4°	17°	9°	Body sets	Rigid	At bottom	Bottom	Rigid	Bottom	Bottom	Bottom
13	10	4	17	9	Rigor jump	Jumps	Sinks	Rigid	Rigid	Rigid	Rigid	Rigid
15	6	5	16	11	Swims up	Swims up	Sinks	Prone	Rigid	Rigid	Rigid	Rigid
15	10	4	17	10	Swims up	Sinks	Sinks	Sinks	Prone	Prone	Prone	Prone
16	6	4	16	9	Body sets	Body sets	Sinks	Bottom	Bottom	Bottom	Bottom	Bottom
17	2	4	17	9	Swims	Rigid	Extended	Jumps	Swims	Rigid	Rigid	Rigid
16	6	4	15	9	Body sets	Kicking	Sinks	Bottom	Prone	Prone	Prone	Prone
17	2	4	15	12	Swims up	Swims up	Swims up	Sinks	Prone	Prone	Prone	Prone

TABLE 13

Responses of normal frogs to total immersion in water 4°C. to 5°C. Entire skin normal. Observation period of 2 minutes divided into 8 sections of 15 seconds each

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS	15 SECONDS
		Cold bath	Frog									
			Before bath	After bath								
18	1	4°	18°	10°	Swims	Floats	Floats	Floats	Sinks	Bottom	Bottom	Bottom
19	1	4	18	9	Swims	Floats	Floats	Floats	Floats	Floats	Sinks	Bottom

one was the same extension of the toes, and stretching of the web accompanied in some frogs by a spasmodic upward jerk of the leg as soon as the tip (table 14) of the toe touched the water (no. 23, exp. 2, table 15). Two frogs had persistently long reaction periods of two and eight seconds, but a very short interval was typical of the cold response.

After a dependable cold response had been shown to occur, further steps were necessary to prove that the cold receptors were in the skin. Their location in its superficial layers had already been suggested by the short reaction interval. In experiments recorded in table 15 responses to cold were invariably eliminated by a half-hour cocaine treatment, whereas this only retarded the heat reaction. The records of the alternate stimulation of the normal foot and the cocained foot by water at 2°C. showed a clock-like regularity in the response of the normal foot.

TABLE 14

Response of foot to water at 2°C. Feet normal. Reaction allowance, 30 seconds. Stimulation period, 2 minutes

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY WATER	REACTIONS	
		Room	Bath water	Frog		Right foot	Left foot
15	13	25°	14°	13°	2°C.	Stretched, rigid, kicked	Stretched, web spread, kicked
15	13	25°	14°	13°	2°C.	Rigid, stretched, flexed	Rigid, stretched, flexed
17	5	25°	14°	14°	2°C.	Stretched, raised	Stretched, web spread
17	5	25°	14°	14°	2°C.	Stretched, raised	Stretched, web spread
20	2	18°	20°	18°	2°C.	Jerked as toe touched	Jerked as toe touched
20	2	18°	20°	18°	2°C.	Stretched, rigid, web spread	Stretched, web spread

At what temperature does the skin become responsive? A definite beginning point (39° to 43°) has already been established for the heat response, but investigations of the cold limitations have not yet been mentioned.

By previous tests the skin had been unresponsive to 25°C. Tests with decreasing cold were begun at this degree therefore and the temperature was decreased with intervals of 5° down to 1°C. The right feet were desensitized in order to further demonstrate the possibility of eliminating the cold sense from the skin. The right and left foot were alternately stimulated but, except

TABLE 15

Responses of foot to water at 2°C. Right foot cocained 30 minutes. Reaction allowance, 30 seconds. Stimulation time, 2 minutes

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.			STIMULATED BY WATER	REACTIONS	
		Room	Bath water	Frog		Right foot	Left foot
13	15	17°	16°	14°	2°C.	None	Toe stretched, web spread
13	15	17°	16°	14°	2°C.	None	Stretched, body twisted
13	15	17°	16°	14°	2°C.	None	Stretched feebly, fatigued
15	15	15°	15°	14°	2°C.	None	Stretched, web spread
15	15	15°	15°	14°	2°C.	None	Leg stretched
15	15	15°	15°	14°	2°C.	None	Leg stretched, fatigue
17	7	19°	17°	14°	2°C.	None	Leg stretched, web spread
17	7	19°	17°	14°	2°C.	None	Leg stretched, web spread
17	7	19°	17°	14°	2°C.	None	Leg jerked
17	7	19°	17°	14°	2°C.	None	Leg stretched, web spread
17	7	19°	17°	14°	2°C.	None	Leg stretched, jerked
17	7	19°	17°	14°	2°C.	None	Leg stretched, fatigue
23	2	20°	18°	16°	2°C.	None	Jerked, web spread
23	2	20°	18°	16°	2°C.	None	Jerked, web spread
23	2	20°	18°	16°	2°C.	None	Jerked, jumped

in one case, there was no response till the temperature was reduced to 10°C. (table 16).

Between 35°C. to 43°C. and 10°C. to 15°C. there was a range of temperature to which the frog's skin did not respond. This range was limited on one side by the threshhold stimulus for heat and on the other by that for cold. The cold as well as the heat receptors have been proved to lie in the skin. The response to cold has its own peculiar characteristics, also a shorter reaction time and an earlier exhaustion point.

Independence of the responses of cold and heat. Cold and heat sensation are separable by exhaustion of the cold receptors and by treatment with cocaine. The right foot was each time immersed for twenty minutes in the solution of cocaine and the right and left feet were then stimulated by water at 2°C. and

TABLE 16

Responses in seconds of the foot to decreasing temperatures. Right foot cocained 30 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.				REACTIONS	
		Room	Bath water	Frog	Stimulus water	Right foot	Left foot
28	2	20°	15°	15°	15°	∞	∞
28	2	20°	15°	15°	10°	∞	1 toes extended, web spread
28	2	20°	15°	15°	5°	∞	1 web spread feebly
28	2	20°	15°	15°	1°	∞	1 toes extended, web spread
31	1	22°	15°	15°	15°	∞	∞
31	1	22°	15°	15°	10°	∞	∞
31	1	22°	15°	15°	5°	∞	1 jerked as toe touched water
31	1	22°	15°	15°	1°	∞	1 jerked as toe touched water
17	11	20°	15°	16°	15°	∞	∞
17	11	20°	15°	16°	10°	∞	11 foot lifted, web spread
17	11	20°	15°	16°	5°	∞	18 foot lifted, web spread
17	11	20°	15°	16°	1°	∞	∞
17	12	21°	25°	14°	15°	∞	∞
17	12	21°	25°	14°	10°	∞	1 toes extended, web spread
17	12	21°	25°	14°	5°	∞	1 jerked
17	12	21°	25°	14°	1°	∞	1 toes extended, web spread

45°C. (table 17). Responses to cold entirely ceased; those to heat remained vigorous with a lengthened reaction time.

Independence of responses to touch and cold. The ease with which the sense of touch disappears from the skin has already been mentioned in connection with touch and heat.

In that case responses to touch failed after the foot was immersed in cocaine 10, 15, 20 and 25 minutes, but reaction to water at 45°C. remained retarded but vigorous. In the same

manner as with touch and cold, touch was very easily eliminated, and by reducing the cocaine treatment the cold sense could be preserved (table 18) in a very effective condition.

Independence of responses to acid and cold. The sensation of cold was separated from the chemical sense in the skins of twelve different frogs. After thirty minutes of treatment with cocaine on the right foot the feet were alternately stimulated as usual.

TABLE 17

Responses of the foot to heat and cold. Right foot cocained 20 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = *no response*

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.				REACTIONS	
		Room	Bath water	Frog	Stimulus water	Right foot	Left foot
5	15	15°	15°	14°	2°	∞	1 toes stretched
5	15	15°	15°	14°	45°	13 jerked	13 jerked
17	6	19°	19°	19°	2°	∞	1 toes stretched, web spread
17	6	19°	19°	19°	40°	25 jerked	5 jerked
17	6	19°	19°	19°	45°	10 jerked	6 jerked
17	6	19°	19°	19°	2°	∞	1 stretched, web spread
17	6	19°	19°	19°	40°	20 jerked	17 jerked
21	5	19°	16°	16°	2°	∞	1 web spread
21	5	19°	16°	16°	40°	17 jerked	10 quiver, jerked
24	6	20°	16°	16°	2°	∞	3 stretched
24	6	20°	16°	16°	40°	9 jerked	6 jerked

The cold response was easily obliterated by the cocaine, but the acid caused a sharp upward jerk at long or irregular reaction intervals. This response itself seemed to differ little from that of the normal foot.

Independence of responses to pain and cold. Pain responses were produced by pricking the skin of the frog on the side of the fifth toe—a procedure that produced more regular results than when the web was similarly stimulated. Care was always taken that the needle did not go into the deeper tissues. The foot was

TABLE 18

Responses in seconds of the foot to touch and cold. Right foot cocained 10 to 25 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.				LENGTH OF TUBE USED FOR SHOT	COCAINE TREATMENT	REACTIONS	
		Room	Bath water	Frog	Stimulus water			Right foot	Left foot
						inches	*minutes*		
23	18	22°	18°	17°		5	25	∞	1 lifted
23	18	22°	18°	17°	2°		25	6 lifted	2 lifted
38	20	21°	16°	17°		5	15	∞	1 lifted
38	20	21°	16°	17°		5	15	∞	1 lifted
38	20	21°	16°	17°	2°		15	1 lifted	10 lifted
39	32	21°	17°	18°		5	25	∞	1 jerked
39	32	21°	17°	18°	2°		25	1 stretched	1 jerked
40	1	20°	17°	19°		5	15	∞	1 lifted
40	1	20°	17°	19°	2°		15	1 shiver	1 lifted
41	1	22°	18°	19°		5	10	∞	1 jiggle
41	1	22°	18°	19°	2°		10	1 web spread	1 jerked

TABLE 19

Responses in seconds of the foot to acid and to cold. Right foot cocained 30 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.				ACID STIMULUS HCl	REACTION	
		Room	Bath water	Frog	Stimulus water		Right foot	Left foot
						per cent		
24	7	23°	16°	18°	2°		∞	3 body twisted
24	7	23°	16°	18°		0.5	15 jerked	2 jerked
24	8	18°	15°	16°	2°		∞	1 toes extended jerk
24	8	18°	15°	16°		0.5	1 jerked	1 jerked
28	3	19°	16°	16°	2°		∞	1 web spread
28	3	19°	16°	16°		0.5	20 jerked	1 jerked
32	1	22°	15°	15°	2°		∞	1 web spread
32	1	22°	15°	15°		0.5	15 jerked	3 jerked

supported when being pricked and by putting the needle only through the side surface it was thought that this was avoided. Twelve frogs were stimulated by pricking and by water at 2°C. Reaction to cold was eliminated by cocaine treatment in as short a time as ten minutes. No effect upon the pain response or its reaction could be discerned.

TABLE 20

Responses in seconds of the foot to pain and cold. Right foot cocained 10 to 30 minutes. Left foot normal. Reaction allowance, 30 seconds. Stimulation time, 2 minutes. ∞ = no response

NUMBER OF INDIVIDUAL	NUMBER OF EXPERIMENT	TEMPERATURES IN DEGREES C.				STIMULATED BY NEEDLE	COCAINE TREATMENT	REACTIONS	
		Room	Bath water	Frog	Stimulus water			Right foot	Left foot
							minutes		
23	1	20°	17°	18°	2°		30	∞	1 spread, lifted
23	1	20°	17°	18°		Pricking	30	1 jerked	1 jerked
43	1	21°	15°	14°	2°		20	∞	1 web spread
43	1	21°	15°	14°		Pricking	20	1 jerked	1 jerked
23	2	20°	18°	17°	2°		20	∞	1 web spread
23	2	20°	18°	17°		Pricking	20	1 jerked	1 jerked
44	1	21°	15°	16°	2°		20	∞	∞
44	1	21°	15°	16°		Pricking	20	1 jerked	1 jerked
23	1	20°	15°	16°	2°		15	∞	1 web spread
23	1	20°	15°	16°		Pricking	15	1 jerked	1 jerked
17	1	21°	16°	17°	2°		10	∞	1 stretched
17	1	21°	16°	17°		Pricking	10	1 jerked	1 jerked

DISCUSSION AND RESULTS

A temperature sense is easily demonstrable in the frog's skin. There is a response to heat characteristic in form and reaction time. The lowest degrees of heat which stimulate the skin lie somewhere between 35°C. and 41°C. If the skin be stimulated by water increasing in heat by 5°C. at each stimulation from 30°C. to 50°C., the first response may be expected at 35°C. If the same series is followed except that the heat be increased by 1°, the first response may occur at 40°C. or 41°C. The skin responds to the higher degrees of heat with great regularity. As the heat is increased from 35°C. to 50°C. the reaction time decreases with more or less regularity from long intervals (25, 15, 12 seconds) to short ones (2, 1 second).

It will be remembered that some of the early workers maintained (Goltz, '69; Heinzmann, '72) that if reflex frogs were stimulated gradually enough with increasing heat they could be subjected to considerable warming without resistance. My investigations show only a slight agreement with them which has been mentioned. It has not been possible to stimulate the foot with increasing heat beyond 43°C. without response, even when the frog's foot was suspended in a beaker of water at 20°C. and the heat almost imperceptibly increased by an inflow of warm water. The long reaction time of the heat response agrees with v. Frey's contention that the heat receptors are in the deeper and the cold receptors in the more superficial layers of the skin.

It has been possible to isolate the temperature sense from the tactile and chemical. This has been done by treatments with 1 per cent solution of cocaine. Crozier ('16) used this method in separating the tactile and chemical senses, and by it Cole ('10) eliminated response to pain, but preserved sensitiveness to taste. The separation of temperature from other senses gave the following results. Response to acid (0.5 per cent hydrochloric) persisted beyond response to heat. Pain persisted beyond heat; heat and cold beyond touch. With the thermal and chemical stimulations care has been taken to immerse the same amount of surface. It has of course not been possible to make any

equivalence between chemical and tactile stimuli or degrees of heat and cold. Granting this necessary inaccuracy, 45°C. heat and 2°C. cold were selected as sufficient extremes to be set against each other.

There is a definite cold sense present in the frog's skin. When the foot was immersed in water of decreasing temperatures, the first responses occurred at 10°C. Contrasted with that of heat, the interval between stimulation and response was an inconsiderable period and could not be accurately taken with a stop-watch. The responses to cold were of two types, a sudden rigidity of the muscles of the leg, with a spreading of the toes and web, or an upward jerk instantly following the contact of the toes with the water. The latter action was less frequent and usually occurred after stimulation by severe cold or in unusually sensitive frogs. Such responses differed from heat responses only in the length of the reaction time.

The sense of cold may be wholly eliminated by cutting the nerve, removing the skin, or by cocaine treatment. It can be shown to be independent of heat, and the tactile and chemical senses by the same treatment. In such comparisons sensation to cold disappears, acid remains; cold disappears and heat and pain remain, but cold remains while touch is eliminated.

The frog's skin is indifferent to temperature of 10°C. or 15°C. to 35°C., whether the stimulation be made by gradual increases or whether it be given suddenly at one selected degree.

SUMMARY

The skin of the frog contains well-defined receptors for heat and for cold. The heat receptors have a comparatively long reaction time. The heat receptors are stimulated by 39°C. to 43°C.; the cold receptors at 10°C. This response is immediate and becomes more vigorous as the cold is increased. The typical response to heat is an upward jerk of the leg. The typical response for cold is a rigidity and tenseness of the muscles, but there may be an upward jerk similar to that of the heat response. Responses to heat and cold may be separated from each other and from the tactile and chemical senses.

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Resumen por el autor, S. R. Detwiler.

Experimentos sobre la transplantación de los miembros en Amblystoma.

Nuevas observaciones sobre las connexiones nerviosas periféricas.

El cambio de posición del rudimento del miembro anterior de Amblystoma en un número determinado de segmentos posteriores o anteriores a su posición normal no afecta del mismo modo el cambio correspondiente de la contribucion del nervio segmentario al plexo braquial. Existe una marcada tendencia en el miembro transplantado a recibir inervación del nivel del miembro normal en la médula. Cuando se transplanta el miembro en una positión anterior de dos o tres segmentos las porciones distales de los nervios del miembro normal crecen anteriormente, en contra de la oposición mecánica de los miotomos en vías de desarrollo, con el fín de efectuar una conexión funcional con el apéndice heterotópico. La posición y extensión del rudimento transplantado determinan solamente hasta cierto punto el número de nervios segmentarios que contribuyen al plexo. Las pruebas acumuladas en estos y en previos experimentos (Detwiler, '20) indican que existe una relación de desarrollo entre el miembro y sus nervios normales, la cual es más íntima en carácter que cualquier otra asociación semejante entre estos nervios y otras estructuras. La función de los miembros transplantados está condicionada por cuatro factores principales: I) La estructura incompleta de la cintura escapular; 2) las deficiencias musculares; 3) la inervación periférica defectiva, y 4) las conexiones defectuosas dentro del sistema nervioso central. La función más completa de los miembros transplantados que reciben nervios del nivel típico del miembro se atribuye al hecho de estar en conexión con mecanismo central de reflejos adecuado para la motilidad normal. Como apéndice al trabajo el autor incluye una consideración teórica de los estímulos que juegan papel en las conexiones periféricas normales.

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EXPERIMENTS ON THE TRANSPLANTATION OF LIMBS IN AMBLYSTOMA

FURTHER OBSERVATIONS ON PERIPHERAL NERVE CONNECTIONS

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THIRTY-TWO FIGURES

INTRODUCTION

In a recent paper dealing with the function and peripheral innervation of transplanted limbs (Detwiler, '20), reference was made to the striking tendency on the part of the normal limb nerves to supply the appendage when the latter was transplanted a considerable distance away from its typical site. In these experiments it was found that shifting the anterior limb a given number of body segments caudal to its normal position on the same embryo did not effect a corresponding shifting of the segmental nerves contributing to its plexus. Limbs transplanted caudally the distance of four and five body segments received one or more nerves from the original limb level of the cord (Detwiler, op. cit., table 2, p. 133).

The facts were also brought out that transplanted limbs which received innervation from the normal limb level of the cord functioned more perfectly than did those whose segmental nerves were derived from the post-limb level, and that the limbs showed a gradually increasing loss of function as they were transplanted farther and farther away from the normal situation. This was attributed to diminution of connections within the central nervous system rather than to a corresponding decrease in effective peripheral innervation or to structural deficiencies within the limb and the shoulder-girdle. The cumulative evidence suggested that the more perfect function of limbs whose

nerves were derived from the original limb level of the cord had its explanation in the fact that the appendage in such cases was connected with a central nervous mechanism adequate for normal motility.

The remarkable increased growth of the limb nerves and the entirely new pathways which they followed to reach their normal terminal end organ (limb) when the latter was transplanted considerable distances caudal to its normal position strongly suggested the possibility that the limb might exert some directive influence upon the segmental nerves contributing to its plexus.

Of considerable significance were the facts that the nerves of the limb level, especially the fourth and fifth, grew greater distances to meet the transplanted limb than did those coming from the more caudal segments, and that the farther away from the normal level the limb was transplanted, the less was its liability to receive nerves from segments situated anterior to the position of the limb, and the greater its tendency to receive nerves from segments corresponding to the position occupied by the limb.

Experiments on various anuran forms (Braus, '05, and Harrison, '07) have shown, in general, that when a limb is transplanted to an abnormal (heterotopic) position it becomes innervated from that part of the central nervous system of the host corresponding to the position occupied by the implanted limb rudiment. In the majority of these experiments the rudiment was transplanted at a stage when the principal nerve paths were already laid down. Accordingly, when the wound in the host was made for the reception of the transplant, the terminal branches of the nerves in that region were severed and the implanted rudiment was placed in close apposition to the cut ends. The possibility of the limb's exerting any directive influence on the nerves could hardly be tested in such cases, since, as has already been pointed out, the limb buds were placed in the direct pathway of already formed spinal nerves whose ends were severed in preparing the wound, and it is to be expected that these nerves would continue their growth into the rudiment so placed.

In the urodele, Amblystoma punctatum, the forelimb rudiment becomes localized quite early, even before the closure of the

medullary folds (Detwiler, '17, '18), and it can be transplanted prior to the period when outgrowth of the spinal nerves begins. Here it is possible, therefore, to test whether or not the transplanted end organ (limb) exerts any directive influence on the normal limb nerves at a time when the latter undergo their initial outgrowth.

The fact that limb nerves made connections with their proper end organ when the latter was transplanted four and five segments caudal to the normal site scarcely seemed explainable on purely mechanical grounds any more than proper selective peripheral connections under normal conditions of development can be explained solely by mechanical agencies. These nerves did not terminate at the limbless area as they did upon simple extirpation of the limb, but they continued their growth caudally until the heterotopic limb was reached and connections were made. The caudoventral elongation of the myotomes undoubtedly acts as a mechanical factor in directing the nerves in this same general course, since they lie in grooves between the elongating muscle segments.

We might conceive of the extended caudal growth of these nerves as due in part to an insufficient number of muscles in the general limbless region for the accommodation of all of their axones. The non-supplied fibers, being directed by the caudoventral elongation of the myotomes, would continue to grow until the limb muscles were reached and connections were made. An interpretation of this nature, however, would not explain why the nerves should finally enter the limb when they do reach it, nor why they should take priority over the nerves coming from segments of the cord corresponding to the position now occupied by the transplanted limb. This evidence, pointing to the directive influence of the transplanted limb upon its normal nerves, suggested the experiments which are taken up in the present paper.

ANATOMICAL

Since these transplantation experiments have been carried out from the standpoint of the nerve connections and functional behavior of the limbs, it becomes necessary, for proper discussion of the results, to consider briefly the anatomical factors involved in the regulation of the motility of the heterotopic appendage. Four such factors have been discussed previously (Detwiler, '19, '20):—1) the completeness of the shoulder-girdle in the heterotopic position, 2) the degree of differentiation of the shoulder and limb muscles, 3) the completeness of peripheral nerve connections with the above muscles, and, 4) the character of the connections within the central nervous system.

1. Shoulder-girdle

Since the shoulder-girdle has the character of a mosaic (Detwiler, '18), its degree of development in the transplanted position is variable, depending on the size of the graft and the region from which it is taken. When a typical limb rudiment is transplanted (fig. 1), only the tissue normally developing into the more central portion of the girdle is included. The localized rudiments of the more outlying portions of the girdle (suprascapula and the ventral portion of the coracoid) lie beyond the limits of the tissue typically included in the limb graft, and consequently undergo development in situ following excision of the transplant. The girdle which develops in the heterotopic position is always reduced in size and is qualitatively incomplete. There is considerable evidence in many cases, however, to show that compensatory hyperplasia from the dorsal and ventral portions of the reduced heterotopic girdle takes place as the larva matures, so that the final size of the girdle may almost equal that of the normal. Such cases will be referred to later.

No conclusive evidence has been obtained from transplantation experiments in Amblystoma to show that the girdle rudiment in this form, in spite of its developmental intimacy with the limb, constitutes a totipotent system such as the limb itself is. Braus ('09, p. 271), however, maintains that in Bombinator the girdle, like the limb, constitutes an equipotential restitution system.

Because of the mosaic nature of the girdle and the subsequent variability in its degree of development in the transplanted position, the motility of the attached appendage is markedly affected by developmental deficiencies in this structure.

2. Shoulder muscles

In a previous description of the shoulder muscles in larval Amblystoma (Detwiler, '20, p. 123) it was pointed out that of the thirteen shoulder muscles which typically develop, nine only connect the shoulder with the limb. The remaining four serve for anchorage of the shoulder and are of myotomic origin. The muscles connecting the shoulder with the extremity are of somatopleural origin, as is the limb itself, and their rudiments are localized in the tissue included in a typical limb disc (fig. 1). Consequently, when the limb rudiment is transplanted, these muscles develop in the heterotopic position (Detwiler, op. cit., pp. 128 and 136). Their nerve supply is derived from the brachial plexus, and although all may be differentiated in the heterotopic position, their nerve supply is by no means constant and many cases develop in which these muscles receive defective nerve supply or are completely lacking in innervation—a condition which markedly affects the degree of motility of the transplanted extremity. The incompleteness of their development secondarily accompanying the smaller area for attachment in the reduced girdle also serves to limit the extent of movements of the extremity on the shoulder.

3. Brachial plexus

The brachial plexus is normally derived from the ventral rami of the third, fourth, and fifth spinal nerves (fig. 4). The developmental evidence indicates that, under normal conditions, these outgrowing nerves effect connection with the limb rudiment when it occupies its maximal extent (anterior border of the third myotome to the posterior border of the fifth, as shown in figure 1) and that convergence of the nerves and plexus formation results secondarily from the concentration of the limb rudiment

into the definitive limb bud which centers ventral to the fourth myotome. From this it would appear that the number of segmental nerves entering the plexus would be determined by the extent of the limb rudiment at the time when initial connections are made.

It was Fürbringer ('79) especially that called attention to the fact that the nerve plexus from which a limb is supplied might in two cases have a different segmental origin, yet the distribution of the limb nerves arising from the plexus might be exactly the same in each. Both Fürbringer (op. cit.) and Gegenbaur ('98), who closely held to the idea that muscle and nerve form an inseparable unit, admitted the difficulty of satisfactorily explaining such segmental variations. From the results of his own experiments, Harrison ('07) interpreted segmental differences as due to the position and extent of the limb rudiment at the time when initial connections of the nerves were made, this serving as an index of the number of nerves contributing to the plexus. From the identity of intrinsic distribution, regardless of metameric origin, Harrison concluded that the mode of segregation and the growth of the structures within the limb determine the specific intrinsic nervous pattern.

From previous observations (Detwiler, '20) and from the results obtained in the present experiments, considerable evidence has accumulated to show that the position and extent of limb rudiments lying beyond the confines of the normal position are not the only factors in determining the source of origin of the nerves contributing to its plexus. This question will be more fully considered in the discussion.

4. *The first and second spinal nerves*

A consideration of the normal pathway of the ventral rami of the first and second spinal nerves is herewith given, since the hypobranchial region is involved in the present experiments.

The course and terminal connections of the ventral rami of the first and second spinal nerves in Amblystoma punctatum are found to be very similar to those described by Norris ('13) for

Siren lacertina, and in general accord with the arrangement characteristic of urodeles (Coghill, '02, '06; Drüner, '01, '03; Kallius, '01, and Norris, '13). The ventral rami of the first and second spinal nerves, typically, unite to form the hypoglossal trunk which supplies the hypobranchial musculature. In Amphiuma, however, Norris ('08) described the hypoglossal trunk as being derived solely from the ventral ramus of the first spinal nerve. A résumé of the arrangement of the hypoglossal complex in both anurans and urodeles is given by Black ('17).

In Amblystoma punctatum the first spinal nerve is entirely motor. It divides into dorsal and ventral rami. The latter at first passes caudolaterally through the dorsal axial musculature to the lateral border of the pharynx. Then it curves laterally and ventrally around the pharynx, and at a point just caudal to where the ramus intestino-accessorius X breaks up into its component divisions, it unites with the anterior prolongation of the ventral division of the second spinal nerve. The common trunk thus formed (hypoglossal), after contributing a branch to the ramus intestino-accessorius X, passes anteriorly along the dorsolateral border of the ventrolateral musculature beneath the pharynx. More anteriorly, it runs slightly lateral to this muscle mass and supplies its component segments (m. sternohyoideus). The distal anterior prolongation of the nerve enters the substance of the m. geniohyoideus, in which it travels for a considerable distance, giving off branches. The terminal portion emerges from the m. geniohyoideus and finally breaks up in the m. genioglossus.

The second spinal nerve arises by both dorsal and ventral roots and presents a small ganglion. The proximal part of the ventral ramus passes posteriorly for a short distance through the longitudinal trunk musculature, then passes laterally to the dorsomesial border of the ventrolateral musculature. Here it curves anteriorly, and after supplying motor fibers to the ventrolateral musculature, it unites with the first spinal nerve. According to Norris ('13), the ventral ramus of the second spinal nerve in Siren contributes a branch to the brachial plexus which in this form is made up principally from the third and fourth spinal

nerves. In Amblystoma punctatum there is normally no communicating branch to the brachial plexus which is formed from the ventral divisions of the third, fourth, and fifth spinal nerves (fig. 4).

The above observations concerning the pathways and connections of the first and second spinal nerves are based on a study of serial transverse sections of larvae ranging in age from fifty to seventy days after the closure of the medullary folds. The arrangement of these nerves is not exactly the same in all cases studied, but the variations are of only minor significance.

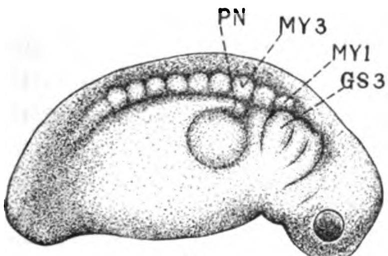
EXPERIMENTAL

The experiments here carried out consisted in transplanting the fore-limb rudiment varying distances anterior to its normal position, with the idea of testing whether the limb nerves could be induced to change their direction of growth and effect connections with the displaced rudiment. Any effort on the part of the nerves to make connections with the limb so placed would necessitate a reversal of their course as compared with that taken by them when innervating limbs placed caudal to the normal site (fig. 5). Under such conditions the nerves, in order to reach their displaced end organ, would meet with considerable opposition, since the differentiating myotomes tend to direct the segmental nerves in a caudoventral pathway. Normal connections made under these conditions would strongly support the idea that there exists a greater attractive influence for the nerves in the developing end organ than in extrinsic structures.

The experiments were carried out upon embryos in the tail-bud state (fig. 1). The circle ventral to the pronephros (*pn*) indicates the position of the limb rudiment. The slightly raised eminence just anterior to the limb rudiment constitutes the gill swellings from which develop the three external gills (cf. figs. 1 and 9). The position of the first and second myotomes with respect to the gill swellings is also seen.

Series A

In the first series of experiments (twenty in number) the limb bud was excised and reimplanted the distance of two segments anterior to the normal position. In preparing the wound for the



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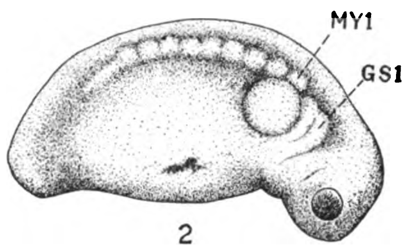

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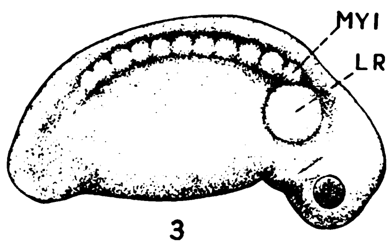

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Fig. 1 Drawing of Amblystoma embryo in the tail-bud stage (stage 29). The circle ventral to the pronephros (*PN*) indicates the position of the fore-limb rudiment. *MY3*, third myotome; *MY1*, first myotome; *GS3*, third external gill swelling. × 10.

Fig. 2 Drawing of Amblystoma embryo, showing the fore-limb rudiment transplanted the distance of two segments anterior to its normal position (cf. fig. 1). The ectoderm and mesoderm of the third gill swelling was removed prior to reimplantation of the limb. *MY1*, myotome 1; *GS1*, first gill swelling. × 10.

Fig. 3 Drawing of Amblystoma embryo following excision of the ectoderm and mesoderm of the gill region and the reimplantation of the right fore-limb rudiment (*LR*) into the excavated area. Denuded limb area covered with indifferent ectoderm. × 10.

reception of the transplant, an area of ectoderm with the underlying mesoderm ventral to the first and second myotomes was excised. This excision involved the tissues normally forming the third gill (fig. 2). The excavated limb area was cleaned of all free mesoderm cells, but was not covered. The results are

given in table 1 A. Four cases of the eleven positive experiments yielded normal results. The limb bud, although originally two segments anterior to the normal and in the region of the third gill, gradually shifted caudally during differentiation and finally assumed the orthotopic (normal) position. The third gill, although late in making its appearance and at first very much smaller than its counterpart, gradually developed to full size. In this series of experiments no normal limbs developed in the heterotopic position. Those which did permanently occupy the

TABLE 1

A. Showing the effects of removing the gill ectoderm and mesoderm from the posterior (third) gill swelling (fig. 2) and transplanting a limb into the excavated area, B. Showing the results of removing the ectoderm and mesoderm from the entire gill swelling (fig. 3) and transplanting a limb into the denuded territory

	NUMBER OF OPERATIONS	POSITIVE EXPERIMENTS	NUMBER OF CASES WITH LIMBS	NORMAL LIMBS	ABNORMAL LIMBS	ABORTIVE LIMBS	FUNCTIONAL LIMBS	NO FUNCTION	REDUPLICATIONS	COMPLETE REGENERATION OF GILLS	ABORTIVE GILLS	ABSENCE OF GILLS
A	20	11	11	4[1]	2	5	6	5	1	6	5	0
B	35	22	22	3	17	1	8	14	3	0	12	10

[1] In all four cases the limb shifted caudally during development and finally occupied the orthotopic position.

The above table does not include one series of forty operations which were made upon unsatisfactory material. The serial case numbers, however, have been preserved (table 2).

transplanted position developed into abnormal appendages, of which 45 per cent were abortive. These limbs, in their initial development, assumed the posture shown in figure 10, but, curiously enough, they soon began to take on the typical posture of the third gill, which failed to develop (fig. 6), and they never fully differentiated into free motile appendages. The first and second gills in some cases developed quite normally, whereas in others they were incomplete and abnormal in appearance.

In case 15 (fig. 7) the limb, except for an imperfectly reduplicated hand, was normal and it functionated to a considerable extent. The third gill was wanting, although the first and second

gills were practically normal in appearance. In case 19 an appendage with a reduplicated forearm and hand developed in place of the third gill. A complete limb which was normal in function regenerated at the original site. The reduplicated appendage exhibited only limited movements in the forearm and hand.

In cases in which complete regeneration of the gills did not occur, the limb retained its transplanted position. Such rudiments, although having a normal aspect at the beginning, soon took on the typical posture of a developing gill, and in all cases distorted and abortive development followed.

In connection with the question of gill development, Ekman ('13, '14) has shown that the factors for the outgrowth of the external gills in various anuran forms (Rana fusca, Rana esculenta, Bombinator, and Hyla) reside entirely in the ectoderm. His experimental results have proved that this ectoderm, which becomes localized before the closure of the medullary folds (stage 1), possesses the properties of self-differentiation. He has also shown that this gill-producing faculty resides not only in the ectoderm of the immediate gill region, but that prospective gill forming potencies extend for a considerable distance beyond the limits of the immediate gill region, particularly in the region of the heart and the pronephros. According to this author, the capacity of the outlying ectoderm to regenerate gills is not the same in all forms—it being higher, for example in Bombinator than in Rana fusca.

The equipotential properties of the gill ectoderm in Amblystoma is secondarily brought out in the present experiments, particularly in series A (table 1), in which it has been shown that after complete extirpation of the tissue of the third gill swelling, complete gill formation occurred in more than 50 per cent of the cases, and that the limb rudiment, in these cases, which originally occupied the region of the third external gill, underwent a caudal displacement so as to lie eventually posterior to the normal gill region.

The difficulty of making successful limb transplantations was likely due to the presence of the inherent gill-producing property

of the ectoderm in the general region surrounding the transplanted rudiment. Developmental conflicts arose between the gill-forming ectoderm and the limb mesoderm, producing not only various distortions in both gills and the limb, but frequently resulting in almost complete suppression of the latter.

Further experiments showed that complete limb differentiation in the gill region could not be expected with any certainty unless complete suppression of the gills was first accomplished by excising much larger areas prior to making the transplantation. Several cases were obtained, however, in which normal limb differentiation did take place, even though one or two atrophic gills did develop.

Series B

In the second series of experiments the ectoderm and mesoderm from the entire gill swelling was removed (fig. 3). The transplanted limb rudiment in these cases occupied a position approximately the distance of three segments anterior to the normal (fig. 3). The excavated limb region in all cases was covered with indifferent ectoderm taken from the caudal portion of another embryo. The results of these transplantations upon the development of the limbs and the gills are summarized in table 1 B. Although the ectoderm and mesoderm from the entire gill swelling was removed, the tabulation shows that 55 per cent of the cases developed abortive and abnormal gills. That these structures develop from tissue lying beyond the confines of the immediate gill swelling is without doubt, and this observation confirms that of Ekmann (op. cit.). Because of the inherent ability of the surrounding ectoderm to produce gills, the percentage of abnormal limbs was high. Even when only one or more abortive gills were formed, the ectoderm surrounding the base of the developing limb frequently migrated out upon the appendage, causing it to become secondarily fused to the side of the body wall (fig. 14). Such limbs, although structurally complete and with considerable nerve supply, were unable to enjoy freedom of movement.

All of the operated animals were kept under daily observation during the first twenty days. A large number in which the limb and the gills showed marked abnormalities were fixed and only the more important cases were kept. Serial transverse sections were made of eight cases ranging in age from twenty-six to sixty days after the operation. The sections were cut 10 μ thick and stained with Ehrlich's haematoxylin and erythrosin.

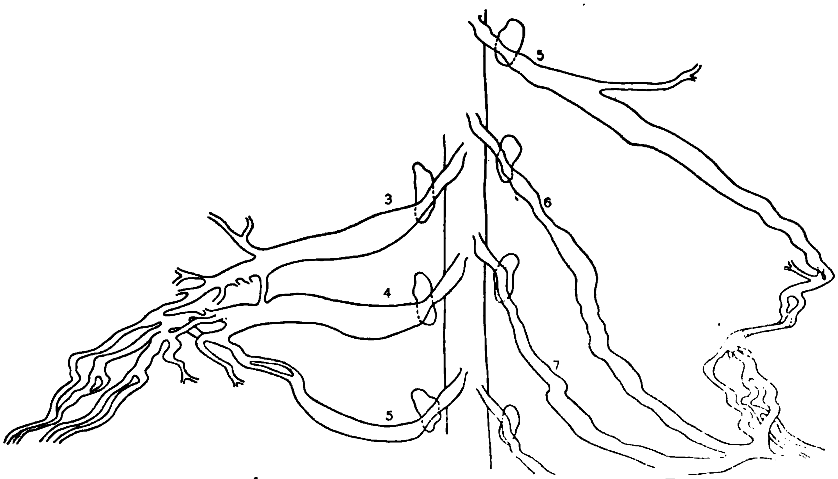

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Fig. 4 Graphic reconstruction of the normal left brachial plexus of Amblystoma larva $AS4_{26}$, preserved sixty-eight days after the operation. × 20. (Detwiler, '20, fig. 13.)

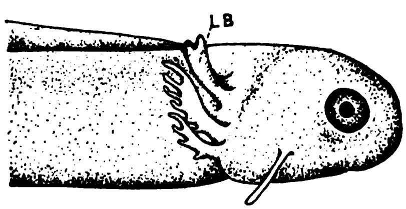
Fig. 5 Graphic reconstruction of the right brachial plexus of case $AS4_{26}$, showing the segmental nerve supply to the right anterior limb when transplanted the distance of four segments posterior to the normal position. × 20. (Detwiler, '20, fig. 10.)

Description of cases

Case $AA2S_{15}$. This experiment was carried out under the conditions described in table 1 A. There was considerable delay in the growth of the limb bud. Only the first and second gills developed, both of which were rather smaller than normal. At fifteen days after the operation the limb rudiment, which occupied the position of the third external gill, took on the charac-

teristic posture of a developing gill and pointed almost dorsally as is seen in figure 6. The limb later changed its orientation and developed into an appendage with considerable freedom of movement, and which was normal except for a reduplication of the digits (fig. 7).

Examination of sections of the above case showed that the glenoid fossa was situated slightly dorsal and approximately the



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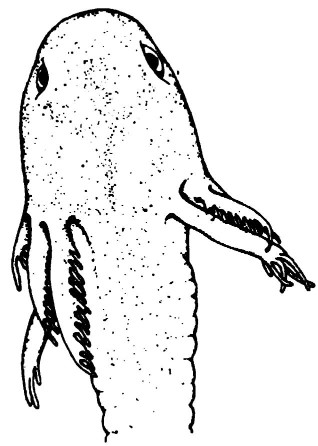

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Fig. 6 Lateral view of case $AA2S_{15}$ drawn fifteen days after operation, showing typical gill posture of the developing appendage (*LB*) which occupies the position of the third external gill (fig. 2). Normal limb posture at approximately same stage is seen in figure 23. × 10.

Fig. 7 Dorsal view of case $AA2S_{15}$ drawn fifty-three days after the operation. The transplanted limb occupies the region of the third external gill (fig. 2). × 5.

distance of two segments anterior to the normal position. The shoulder-girdle was well formed. All of the shoulder muscles which typically develop in the heterotopic position from a typical limb-bud transplantation (Detwiler, '20, p. 136) were present. The m. coracobrachialis longus and the m. coracobrachialis brevis were small and abnormal. The remainder of the shoulder muscles were typical. The musculature of the arm was somewhat deficient on the extensor surface. The limb and shoulder mus-

cles were supplied by a plexus derived from the ventral rami of the second, third, and fourth spinal nerves (fig. 8), the latter two of which are normal limb nerves. It is seen that the distal portions of the nerves have grown anteriorly to effect connection with the heterotopic limb. The typical course of the third and fourth nerves is shown in figure 4. The ventral ramus of the second spinal nerve, which typically unites with that of the first to form the hypoglossal, is contributed almost entirely to the brachial plexus. The completeness of peripheral connection with the shoulder muscles is given in table 3.

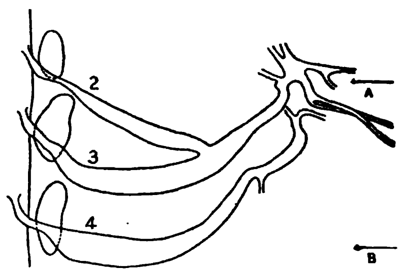


Fig. 8 Graphic reconstruction of the ventral rami of the second, third, and fourth spinal nerves, showing their direction of growth and contribution to the limb plexus in case $AA2S_{18}$. Arrow *A* indicates position of transplanted limb; arrow *B* designates approximate level of normal limb. For normal pathways of third and fourth spinal (limb) nerves (fig. 4). $\times$ 25.

The ventrolateral musculature in this case is almost entirely wanting. The ventral ramus of the first spinal nerve supplies the fragmentary m. sternohyoideus, the anterior prolongation of which is entirely wanting as is the entire m. geniohyoideus and the m. genioglossus. The hyoid cartilage on the operated side was also wanting. The deficient development of the m. sternohyoideus and the complete absence of its derivatives, the m. geniohyoideus and m. genioglossus, indicate that the myotomic rudiments of these muscles were excised in preparing the wound for the reception of the transplant. Lewis ('10) has experimentally shown that the sternohyoid portion of the ventrolateral musculature in Amblystoma is derived from the ventral processes of the first three myotomes, and that, after extirpation of the

first myotome, the anterior portion of this muscle is entirely lacking as is its derivative, the m. geniohyoideus. No mention was made of the genesis of the m. hyoglossus, but from the present experiments, the evidence suggests that this muscle also arises from the anterior segment of the m. sternohyoideus, for when the anterior segments of this muscle are lacking, the m. hyoglossus is also absent.

The ventral portion of the first myotome in Amblystoma is in intimate relation to the gill mass (fig. 1). The deficiencies

9

10

Fig. 9 Left lateral aspect of case $AA2S_{21}$ drawn seven days after the operation. × 10.

Fig. 10 Right lateral aspect of case $AA2S_{21}$ drawn seven days after the operation. The limb bud occupies the region of the second and third external gills. The first gill (*EG1*) is considerably smaller than the normal (fig. 9). × 10.

in the hypobranchial musculature on the operated side indicate that in preparing the wound for the limb rudiment, the ventral portion of the first myotome, as well as a portion of the second, was excised.

Case $AA2S_{21}$. In this experiment, the ectoderm and mesoderm of the entire gill region were excised and the limb rudiment was transplanted into the excavated area, as seen under conditions indicated in figure 3. The denuded limb area was covered with indifferent ectoderm. Seven days after the operation the appearance of an external gill was seen between the heterotopic limb bud and the balancer (fig. 10, cf. fig. 9). At fourteen days

the developing limb bud had rotated dorsally under the influence of ectoderm dorsal to the limb, simulating a condition shown in figure 6. It later assumed typical orientation and developed into an appendage which, although somewhat small, was externally normal in appearance (fig. 11). During later development, the limb gradually shifted caudally so that its final position was scarcely the distance of two segments anterior to the orthotopic position. Movements of the limb on the shoulder were quite defective, owing to incomplete muscular differentiation and imperfect innervation (table 3). The musculature of the

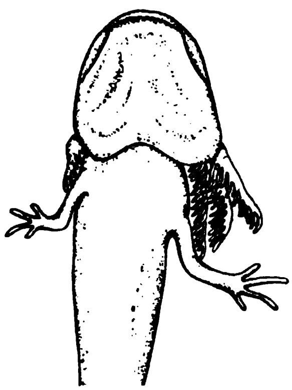


Fig. 11 Ventral aspect of case $AA2S_{31}$ drawn forty-eight days after the operation. Only one small external gill has developed, and the limb, which earlier occupied the region of the second and third external gills (figs. 9 and 10) has migrated caudally to its final position. × 5.

limb itself was well differentiated and received innervation from two nerve trunks (fig. 12), one on the flexor surface and the other on the extensor surface. The shoulder-girdle was typical in shape, although the ventral zone was much shorter than normal, with a correspondingly curtailed development of the coracobrachial and the pectoral muscles. The limb and shoulder were innervated by the ventral divisions of the second and third spinal nerves (fig. 12). The distal portion of the latter, which contributed the main bulk of the nerve supply to the limb and the shoulder had elongated a considerable distance anteriorly. The ventral ramus of the second spinal nerve passed at first caudally

through the dorsal trunk musculature and then almost directly laterally. Before uniting with the third nerve it supplied several branches to the abdominohyoideus musculature. No union was found between the ventral rami of the first and second nerves. The former was found to pass somewhat caudally, then laterally, terminating in the posterior segments of the m. sternohyoideus.

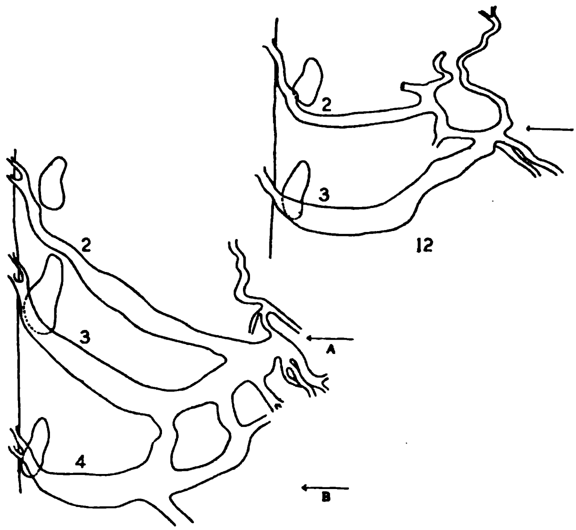


Fig. 12 Graphic reconstruction of the segmental nerve contribution to transplanted limb in case $AA2S_{21}$ (fig. 11). × 25. Arrow designates level of transplanted limb.

Fig. 13 Graphic reconstruction of the segmental nerve supply to transplanted limb in case $AA2S_{24}$. Limb, transplanted into the gill region (fig. 2), has migrated caudally during development, finally occupying a position approximately one segment anterior to the normal position. × 25. Arrow *A* indicates position of transplanted limb; arrow *B* designates normal limb level.

A typical anterior prolongation of the hypoglossal nerve was lacking in this case, as were also the anterior segments of the m. sternohyoideus and their derivatives (m. geniohyoideus and m. genioglossus).

Case $AA1S_{24}$. Although in this experiment the limb rudiment was transplanted into the gill region, as is indicated in figure 3, its final position was only one segment anterior to the normal,

and it is hence classified under series AA1S (table 2). Owing to the early caudal migration of the rudiment in this case, development proceeded normally. Although the limb occupied a position caudal to the gill region, gill formation was almost entirely suppressed, and only one small external gill developed, which, from its position, was taken to be the first.

TABLE 2

Showing the segmental nerve contribution to the right fore limb when transplanted. A. One to three segments anterior to the normal position (series AA1S, AA2S). B.[1] *One to five segments caudal to the normal position (series AS1, AS2, etc.)*

	SERIES	CASES	POSITION OF LIMB		SEGMENTAL NERVE CONTRIBUTION								
			Number of segments anterior to normal position	Number of segments caudal to normal position	1	2	3	4	5	6	7	8	9
A	Normal	1					3	4	5				
	AA1S	3	1				3	4	5				
		24	1			2	3	4					
	AA2S	15	2			2	3	4					
		21	2			2	3	4					
		25	2½			2	3	4					
		73	2			2	3	4					
		78	2½–3		1	2	3						
B	AS1	12		1			3	4	5				
	AS2	5		2			3	4	5				
		12		2			3	4	5				
	AS3	9		3				4	5	6			
		18		3				4	5	6			
	AS4	12		3				4	5	6	7		
		24		4					5	6	7		
		30		4					5	6	7		
	AS5	25		5					5	6	7	8	9
		27		5						6	7	8	9
		30		5						6	7	8	9

[1] The figures in 'B' are taken from a previously published table (Detwiler, '20).

TABLE 3

Showing the shoulder muscles which developed with the transplanted limb, and the presence or absence of their respective nerve supply[1]

SERIES	INDIVIDUAL CASES	MUSCLES																	
		M. procoraco-humeralis		M. subcoraco-scapularis		M. supracora-coideus		M. dorsalis scapulae		M. latissimus dorsi.		M. anconaeus scapularis medialis		M. coracobrachialis brevis		M. coraco-brachialis longus		M. pectoralis	
AA1S. .	24	Present	+	Present	+	Present	+	Present	+	Present	+	Present	+	Present	+	Present	+	Present	+
AA2S.	15	Present	+	(Very small)	−	Present	+	Present	+	(Small)	−	Present	+	Absent	−	(Very small)	?	Present	+
AA2S. .	21	Present	+	(Very small)	?	Present	+	Absent	−	Absent	−	Absent	−	Short	?	(Very short)	−	Short	?
AA2S. .	25	Present	+	Present	−	(Very short)	+	Absent	−	Absent	−	Present	+	Present	?	Present	+	Present	+
A . .	75 {1	Absent	−	Absent	−	Absent	−	Absent	−	Absent	−	Absent	−	Present	+	Present	+	Present	+
	75 {2	Present	+	Present	+	Present	+	Present	+	Present	+	Present	+	Present	+(?)	Present	+	Present	+
AA2S. .	78	Present	−	Present	−	Present	+	Present	+	Present	−	Present	+	Present	+(?)	Present	+	Present	+

[1] + signifies that muscle is innervated; − signifies that muscle lacks innervation.
1 = anterior member of reduplication.
2 = posterior member of reduplication.

Movements of the limb were practically normal. The shoulder and limb muscles were completely differentiated and were well supplied by nerve fibers from the ventral rami of the second, third, and fourth spinal nerves (fig. 13). From this figure it is seen that although the course of the proximal portions of the nerves is very similar to that of the normal limb nerves (fig. 4), their distal portions are continued anteriorly for some distance to the heterotopic limb. The ventral ramus of the second nerve had no connection with that of the first and passed directly to the brachial plexus. The ventral ramus of the first nerve was distributed to the fragmentary m. sternohyoideus, the anterior portion of which was wanting, as were also the m. geniohyoideus and the m. hyoglossus.

In comparing the muscular differentiation and innervation of this case with that of former and subsequent cases (table 3) in which the limb developed in the region of the gills, it is seen that the immediate organic environment of the limb distinctly favors more normal development. Cases in which the transplanted rudiment remained in the gill region and there underwent differentiation showed considerable deficiencies in the development of the shoulder muscles as well as defective peripheral nerve connections (cases $AA2S_{15, 21, 25}$, etc., table 3). The large percentage of defective limbs is likewise shown in table 1 B. The above shows that even though the limb constitutes an equipotential, self-differentiating system, the character of its development is markedly affected by developmental potencies of the region into which it is transplanted. Although the transplanted limb rudiment possesses complete intrinsic potentialities to develop into a normal appendage, it is clearly evident in these cases that the high percentage of abnormalities in the gill region is due to an inhibitory influence resulting from the more potent inherent gill-producing properties of the tissues in the immediate organic environment of the gills which offer a very unfavorable environment for normal limb differentiation. The abnormalities on the part of both gills and limb clearly show developmental conflicts between the two systems.

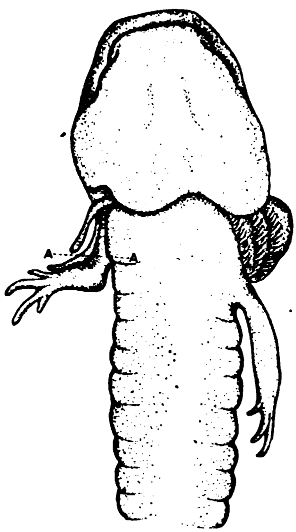
The general results of these experiments indicate that when the major portion of the gill tissue is removed and replaced by an entire limb rudiment, the remaining unremoved portion of the gill tissue, being in its normal organic environment, possesses a relatively greater potency to produce normal gills than does the entire limb system to produce a normal appendage—the latter being in the heterotopic position. When a limb is transplanted into a more passive region of the embryo such as that caudal to its normal position, practically normal differentiation results. For example, limbs reimplanted the distance of three body segments caudal to the normal location yielded 75 per cent normalities (Detwiler, '20). When transplanted the same distance anterior to the normal site, into the active self-differentiating gill region, less than 15 per cent of the cases developed normally (table 1).

Case $AA2S_{25}$. In this experiment (fig. 14) the initial development of the limb was normal. Only one rudimentary gill developed, but the ectoderm in the vicinity of the base of the limb wandered out over its flexor surface forming a permanent ridge (figs. 14 and 15). The caudal border of the proximal portion of the limb was also fused with the ectoderm of the gill, which greatly restricted its motility. Function of the forearm and hand were practically normal.

Serial transverse sections showed that the glenoid cavity was approximately two and one-half segments anterior to the normal position. The shoulder-girdle was abnormal. Its coracoid portion was continuous with the fragmentary coracoid which developed in the orthotopic position from unremoved portions of its rudiment. The suprascapular portion was short and quite thick.

The shoulder muscles were poorly developed and were somewhat defective in nerve supply (table 3), which was derived from the ventral rami of the second, third, and fourth spinal nerves (fig. 16). Although there was an abundant nerve supply from the cord, a survey of the sections shows clearly that the incompleteness of function in this case is due to the abnormal development of the shoulder-girdle, deficiency of its corresponding musculature, and the fusion of its proximal portion with the gill.

The anterior elongation of the distal portions of the limb nerves is seen in figure 16. It is interesting to note that the fourth nerve did not unite with the third until the periphery had been reached by an extended anterolateral growth. The ventral ramus of the second spinal nerve showed no connection with



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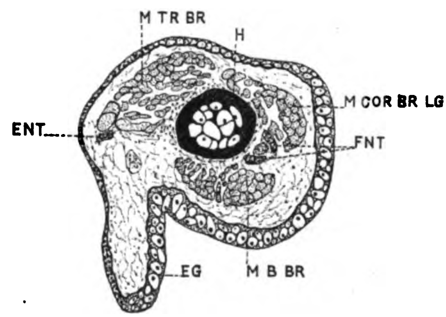

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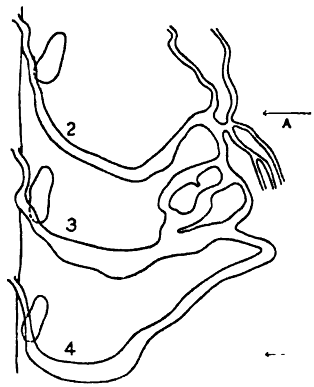
Fig. 14 Ventral view of case $AA2S_{25}$. Limb transplanted into the gill region as indicated in figure 3. Final position of limb, two and one-half body segments anterior to normal level. Animal preserved fifty-one days after the operation. × 5.

Fig. 15 Transverse section of right brachium of case $AA2S_{25}$, at the level *A–A*, figure 14, showing fusion of abnormal gill with radial side of the appendage. × 58. No vascular elements have developed in the anomalous gill. *H*, humerus; *M TR BR*, m. triceps brachii; *M COR BR LG.*, m. coracobrachialis longus; *M B BR*, m. biceps brachii; *FNT*, flexor nerve trunk; *ENT*, extensor nerve trunk; *EG*, ectoderm of gill.

that of the first, and its entire trunk was contributed to the plexus. The hypoglossal nerve was formed solely of the first spinal nerve which, after supplying branches to the somewhat reduced m. sternohyoideus, continued anteriorly to end in the m. geniohyoideus and the m. hyoglossus.

Case AA2S$_{73}$. In this experiment, which was carried out under conditions indicated in table 1 B, the development of external gills was completely suppressed and two limbs of the same laterality developed in the heterotopic position (fig. 17). From external observations on the developing larva it was impossible to determine whether both members of the double limb developed



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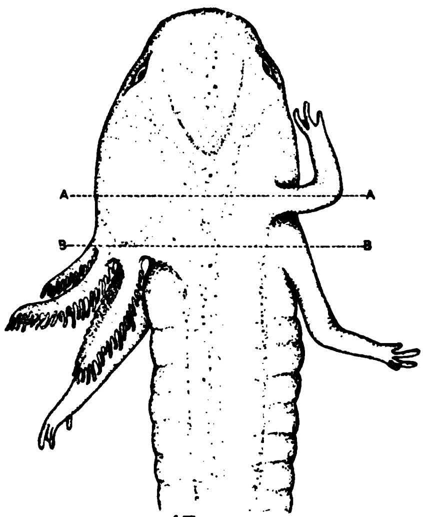

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Fig. 16 Graphic reconstruction of segmental nerve supply to transplanted right anterior limb in case AA2S$_{25}$ (fig. 14). Limb occupies a position approximately two and one-half segments anterior to the normal. Arrow *A* indicates level of transplanted limb; arrow *B* designates normal limb level. × 25.

Fig. 17 Dorsal view of case AA2S$_{73}$, showing absence of gills and presence of two limbs of same laterality developed from anomalous reduplication of the transplanted rudiment. Experiment carried out under conditions indicated in table 1 B (fig. 3). × 5.

from the transplant or whether the caudal member developed from an unremoved portion of the original rudiment. The limb bud which developed into the anterior appendage was the first to appear. This was soon followed by a second bud which developed into the caudal member. The fact that the final position of the latter was approximately two segments anterior to the normal position suggested that it developed from the transplanted rudiment. Moreover, examinations of sections showed that its

shoulder-girdle was developed anterior to the isolated distal portions of the intact suprascapula and coracoid, which always develop in situ after a typical limb-bud excision. Further, the fact that the anterior member lacked a girdle, except for a very fragmentary coracoid, also indicated that the well-developed girdle of the caudal member was developed from the girdle rudiment included in the transplant.

Previous limb experiments on Amblystoma have shown that when supernumerary limbs develop from a single transplant under conditions of normal orientation with respect to the surrounding tissue, the one is always the mirror image of the other (Harrison, '17, '21; Detwiler, '18), i.e., a disharmonic appendage. In the light of these results, the anomalous reduplication in the present experiment, if resulting from a single transplant as the evidence favors, is an unusual phenomenon. Harrison ('21), however, describes several cases with anomalous double-limb formation, both of the same laterality, resulting from a composite rudiment in the orthotopic position. The two limbs in these cases are shown in Harrison's paper (l.c.), 79, figs. 131 and 132. The combinations producing them are shown on page 70 of his paper (combinations 10 and 11).

In the case under consideration, the development of a well-formed branchial bar between the bases of the two limbs might have effected cleavage of the original transplanted rudiment, resulting thereby in the development of the two limbs. It has been shown by Harrison ('18) that, under normal conditions, splitting the limb bud by a vertical or horizontal incision never produces reduplication. There is no apparent reason, however why two portions of a transplanted limb rudiment, if permanently separated, should not develop into two limbs of the same laterality. Such a result is normally produced when a portion of the rudiment is transplanted: one limb will develop at the original site and the other at the heterotopic position.

In the present case the anterior limb developed in the posterior region of the otic capsule. The shoulder-girdle consisted of only a fragmentary coracoid. This was fused dorsally with the cartilaginous capsule of the ear, which, together with the small

coracoid, was molded into a typical shoulder-joint (fig. 30). This figure represents a transverse section at the level A—A in figure 17. A muscle mass connected the coracoid with the humerus, but the individuality of the muscles could not be determined. The extensor musculature of the limb was well developed and supplied by a small nerve trunk. The flexor musculature was sparse, but functional. A nerve trunk of considerable size was also found on this surface of the appendage. Considerable

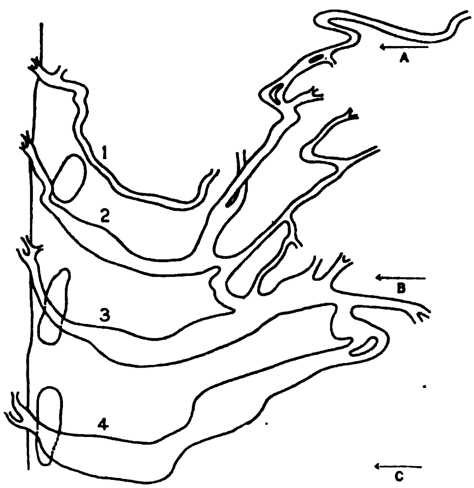

Fig. 18 Graphic reconstruction of the segmental nerve supply to the anterior and posterior members of anomalous limb reduplication in case $AA2S_{73}$. × 25. Arrow *A* indicates position of anterior member; arrow *B* of posterior member; arrow *C* designates normal limb level.

movements in the forearm and hand were observed. The nerve contribution to this appendage was derived from the main portion of the ventral ramus of the second spinal nerve, which had elongated anteriorly a remarkable distance to effect this connection (fig. 18). The base of the caudal limb was situated approximately the distance of two body segments anterior to the normal position. The shoulder-girdle was well formed (fig. 31). Its extreme dorsal portion (suprascapula) was connected with the fragmentary coracoid of the anterior limb by means of a bar of cartilage which was taken to be a modified branchial bar

(fig. 31). The shoulder muscles were typically developed and were supplied by nerves (table 3). The muscles within the limb were normally differentiated and were supplied with nerves of typical intrinsic distribution. The segmental nerve supply to this limb comprised the ventral rami of the second, third, and fourth nerves (fig. 18). The greater part of the nerve supply was derived from the latter two, although a good-sized branch from the second contributed to the plexus. The second nerve, in addition to supplying the anterior limb and a contributing

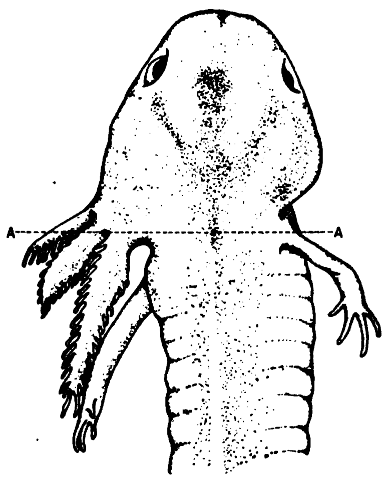

Fig. 19 Dorsal view of case $AA2S_{78}$, showing absence of gills and the position of limb developed from rudiment transplanted into gill region (fig. 3). Animal preserved fifty-nine days after the operation. × 5.

trunk to the posterior appendage, supplied fibers to the m. abdominohyoideus. No communication with the first spinal nerve could be found. In this case the hypoglossal trunk was formed solely from the latter, which exhibited a normal course along the dorsolateral border of the m. sternohyoideus in which it finally terminated. The m. geniohyoideus and the m. hyoglossus were absent on the operated side.

Case $AA2S_{78}$. In this experiment (tables 1 B and 2 A), gill development was also entirely suppressed and the transplanted rudiment developed into a normal appendage (fig. 19). Its

location was somewhat dorsal and approximately the distance of two and one-half segments anterior to the normal site. Examination of sections revealed a well-developed shoulder-girdle. The shoulder muscles were normally present, but within complete nerve supply (table 3), which placed considerable restrictions on the shoulder movements. The muscles of the limb were typically developed. Only one nerve trunk entered the limb, however, and this was situated on the flexor surface. The extensor paralysis which this limb showed and the absence of an extensor nerve trunk have been observed in a considerable number of trans-

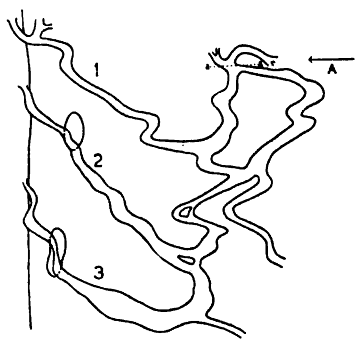

Fig. 20 Graphic reconstruction of segmental nerves supplying transplanted right anterior limb in case AA2S$_{78}$ (fig. 19). × 25. Arrow indicates position of limb and level of section shown in figure 32.

planted limbs. The reasons for this specific deficiency in the presence of muscular differentiation are not yet clear.

The nerve supply to the shoulder and limb was derived from the ventral rami of the first, second, and third spinal nerves. The pathway of these nerves in their contribution to the limb plexus is seen in figure 20. The entire ventral rami of the first and second spinal nerve were contributed to the brachial plexus. The ventrolateral musculature, which was sparse in this case, lacked innervation. A small m. sternohyoideus was present, but the m. geniohyoideus and the m. hyoglossus were entirely lacking. Figure 32 shows the internal configuration of a transverse section at the level *A—A*, figure 19. The ventral ramus

of the first spinal nerve is seen in cross-section. The distal nerve trunk in the vicinity of the limb region is indicated by the line *a–a* in figure 20. This nerve trunk represents a fusion of the distal portions of the ventral rami of the first, second, and third spinal nerves, all of which have grown considerable distances anteriorly to reach the transplanted limb.

Two cases in which normal limbs developed, but which have not been sectioned, are shown in figures 21 and 22. In case

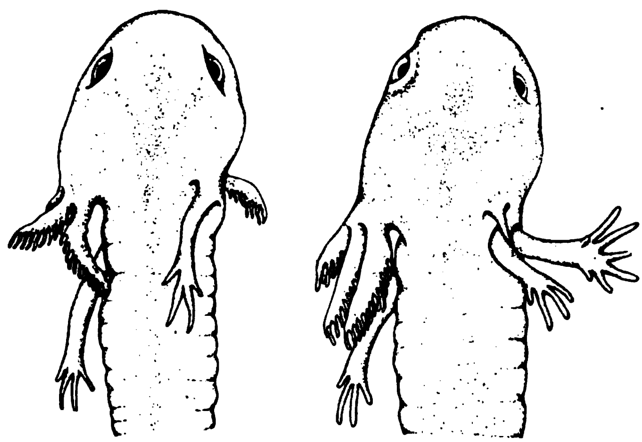

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Fig. 21 Dorsal view of case AA2S$_{50}$, showing presence of normal functionless limb, developed from rudiment transplanted into gill region (fig. 2). Animal preserved forty-four days after the operation. × 5.

Fig. 22 Dorsal view of case AA2S$_{52}$, showing transplanted and regenerated limbs. The base of the former is fused with a strip of gill ectoderm which has grown out along its radial border. Incomplete reduplication of the hand is seen in the regenerated appendage. Animal preserved forty-four days after the operation. × 5.

AA2S$_{50}$ (fig. 21) the limb, although normal in appearance, was practically devoid of function. One small gill developed which was situated farther ventral than normal. In case AA2S$_{52}$ (fig. 22) the transplanted limb was quite normal in appearance. A fold of ectoderm extended out over its base and was fused along the radial side, thus binding the limb close to the side of the body. Just posterior to the transplanted limb there regenerated another limb with reduplication of the hand. Gill development was entirely suppressed.

Histories of characteristic anomalous cases

Inasmuch as quite a large percentage of the experiments resulted in gill and limb abnormalities of similar character, the record of only a few such typical cases are appended.

Case AA2S$_{27}$ (figs. 23 and 24). March 3, 1920. Operation. Rt. limb transplanted into gill region. Conditions as described in table 1 B. Wound covered.

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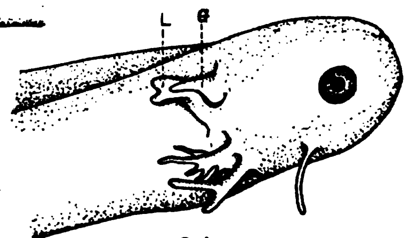

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Fig. 23 Drawing of left lateral aspect of case AA2S$_{27}$. Animal preserved twenty days after the operation. × 10.

Fig. 24 Drawing of right lateral aspect of case AA2S$_{27}$ (cf. fig. 23). The abnormal limb (*L*) has developed from rudiment transplanted into the gill region (fig. 2). A club-shaped external gill (*G*) has developed dorsal to the appendage and has fused with its lateral side. Proximal portion of arm is fused to gill ectoderm and points dorsocaudally. Two small gills are seen ventral to the limb. × 10.

March 29. Completely healed.

April 2. Transplanted limb bud points caudally, as in figure 10.

April 7. Two small gills developing ventral and slightly anterior to limb rudiment.

April 12. Limb points dorsally and is closely applied to the ectoderm of the gill region, as in figure 6.

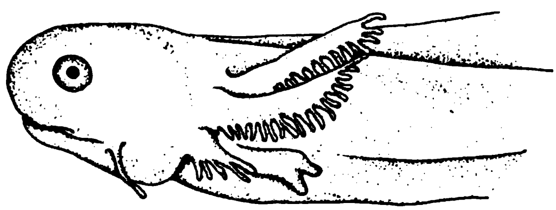
April 16. Distal portion of the limb has rotated laterally and is directed caudally. A flange of ectoderm dorsal to the limb has extended out along the dorsal border of the arm. Two abortive gills situated ventral to the limb.

April 18. Proximal portion of the limb fused to the body wall. Distal portion is free and points caudally. The ectoderm dorsal to the limb has developed into an abortive gill which is fused to the lateral side of the arm (fig. 24).

Case AA2S₅₄. April 3, 1920. Operation. Conditions of experiment as in AA2S₂₇. Wound covered.

April 8. Limb bud developing with normal orientation.

April 12. Developing limb bud points dorsally and is fused to the side of the body. Gill ectoderm extends out along the dorsum of the



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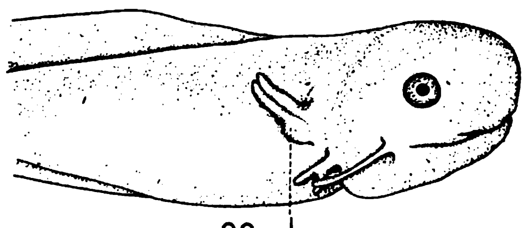

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Fig. 25 Drawing of left lateral aspect of case $AA2S_{54}$. Animal preserved eighteen days after the operation. × 10.

Fig. 26 Drawing of right lateral aspect of case $AA2S_{54}$ (fig. 25). Anterior limb transplanted into the gill region as described in table 1 B. The limb (*B*) is abortive and is fused dorsally with an extension of the gill ectoderm which has migrated out over the extremity. A small atrophic gill has developed ventral to the limb. × 10.

free distal portion. One small gill developing anterior and ventral to the limb.

April 18. Gill ectoderm has completely fused along the dorsum of the limb. Limb very abnormal and reduced to a club-shaped appendage. Small atrophic gill ventral to the limb.

April 22. Animal preserved (fig. 26, cf. fig. 25).

Case $AA2S_{55}$. April 3. Operation. Experimental conditions as in case $AA2S_{27}$. Wound covered.

April 4. Completely healed.

April 9. Limb bud developing with normal orientation.

April 12. Two small gills developing anterior to limb. Limb abortive and is rotated dorsally.

April 20. Posterior (second) gill fused along anterior border of atrophic limb. First gill very small.

April 29. Animal preserved (fig. 27).

Section of the above case shows the presence of several abortive and abnormally developed branchial bars. There is a small limb girdle, the glenoid cavity of which is deeply situated. Only the distal portion of the arm is free from the body. The appendage lacks muscular differentiation. The ectoderm of the fused external gill (fig. 27) is

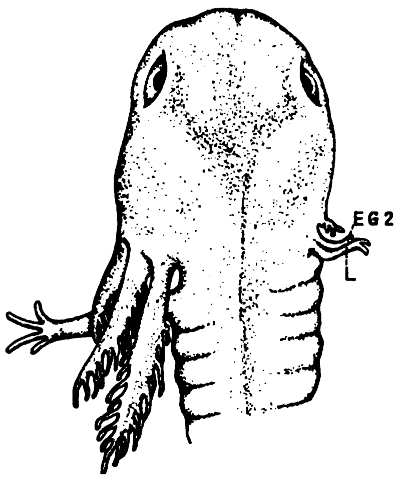


Fig. 27 Dorsal view of case $AA2S_{33}$. Right anterior limb transplanted into gill region as under conditions described in table 1 B. The limb (*L*) is abortive. Two abortive gills are seen anterior to the limb. The second (*EG2*) is fused along the radial border of the limb. Animal preserved twenty-six days after the operation. × 10.

continuous with that covering the extremity. No vascular elements are differentiated.

Case $AA2S_{64}$. April 4, 1920. Operation. Experimental conditions as in case $AA2S_{27}$.

April 5. Completely healed.

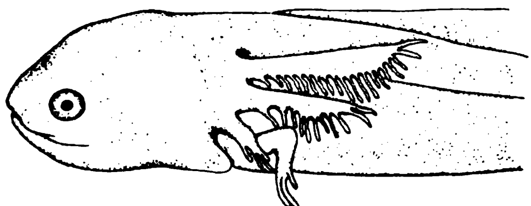
April 10. Limb bud developing with normal orientation. No evidence of developing gills.

April 15. Limb points dorsally and the proximal part is fused to side of body wall. Gill ectoderm fuses along dorsum of the limb. Very small, abortive gills developing ventral to the typical gill region.

April 20. Distal portion of limb club-shaped. Digits fail to develop. Gill ectoderm is connected with limb along its dorsomesial border. Small spur developed on lateral border of arm.

April 25. Animal preserved (fig. 29, cf. fig. 28). Very abnormal, functionless limb which is fused dorsally with an extension of gill ectoderm. Three abortive gills situated ventral to the gill shelf.

The gill-producing potentiality of the outlying ectoderm is well illustrated in the above cases. In all of these experiments the entire ectodermal and mesodermal tissues of the gill swellings were removed prior to implantation of limb, yet one or more gills



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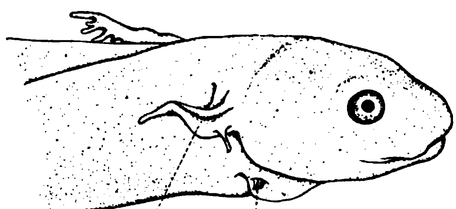

L 29 EG

Fig. 28 Drawing of left lateral aspect of case $AA2S_{64}$. Animal preserved thirty-six days after operation. × 7.

Fig. 29 Drawing of right lateral aspect of case $AA2S_{64}$ (fig. 28). Fore-limb rudiment transplanted into gill region as described in table 1 B. Gill ectoderm dorsal to abortive limb (*L*) has migrated out on the limb and is fused along its dorsomesial surface. Three very small external gills (*EG*) have developed out of the tissue ventral to the normal gill region. × 7.

developed in all four cases. From the results of these cases, as well as from others not reported, the evidence indicates that the ectoderm lying ventral to the typical gill region possesses a relatively stronger gill-forming capacity than does that lying anterior and dorsal. In the majority of cases the gills developed ventral to the gill region in approximation to the general heart area as illustrated in figures 24, 26, and 29. This observation supports the results obtained by Ekman ('13 b, p. 578, vide supra, p. 125).

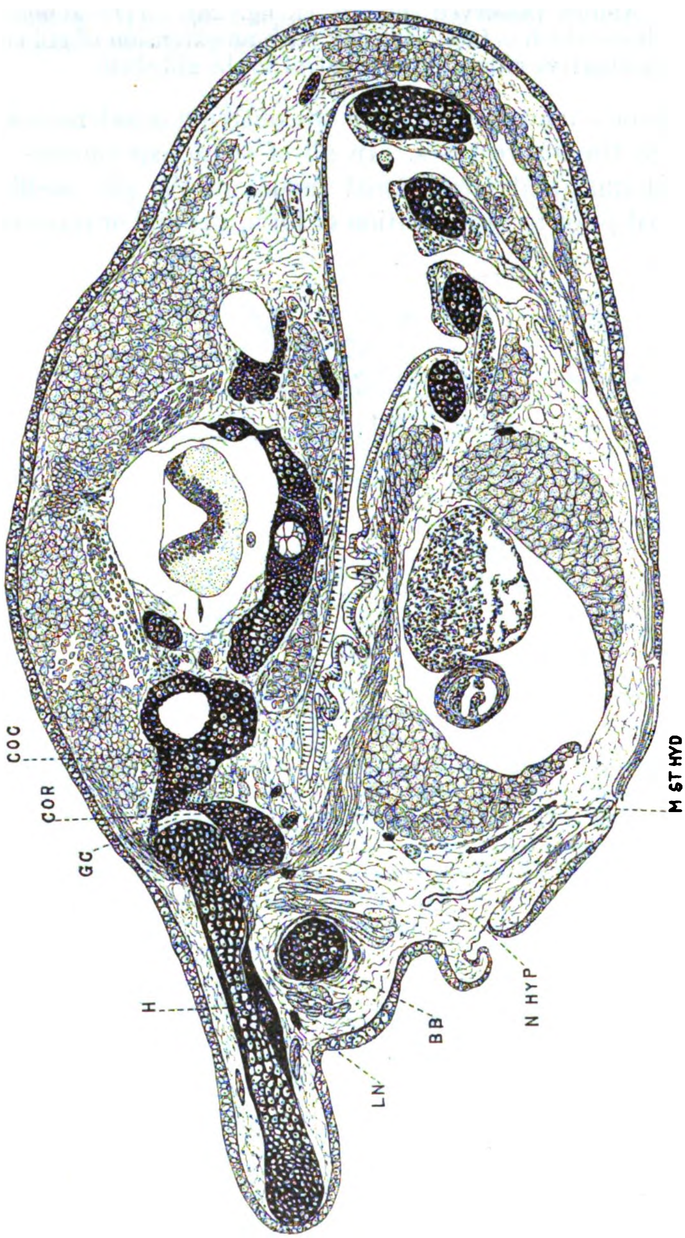

Fig. 30 Drawing of transverse section (no. 394) of case AA2S$_{73}$ at the level *A–A*, figure 17. × 24. *H*, humerus; *GC*, glenoid cavity; *COR*, coracoid; *COC*, cartilaginous otic capsule; *LN*, limb nerve; *BB*, branchial bar; *N HYP*, hypoglossal nerve; *M ST HYD*, m. sternohyoideus.

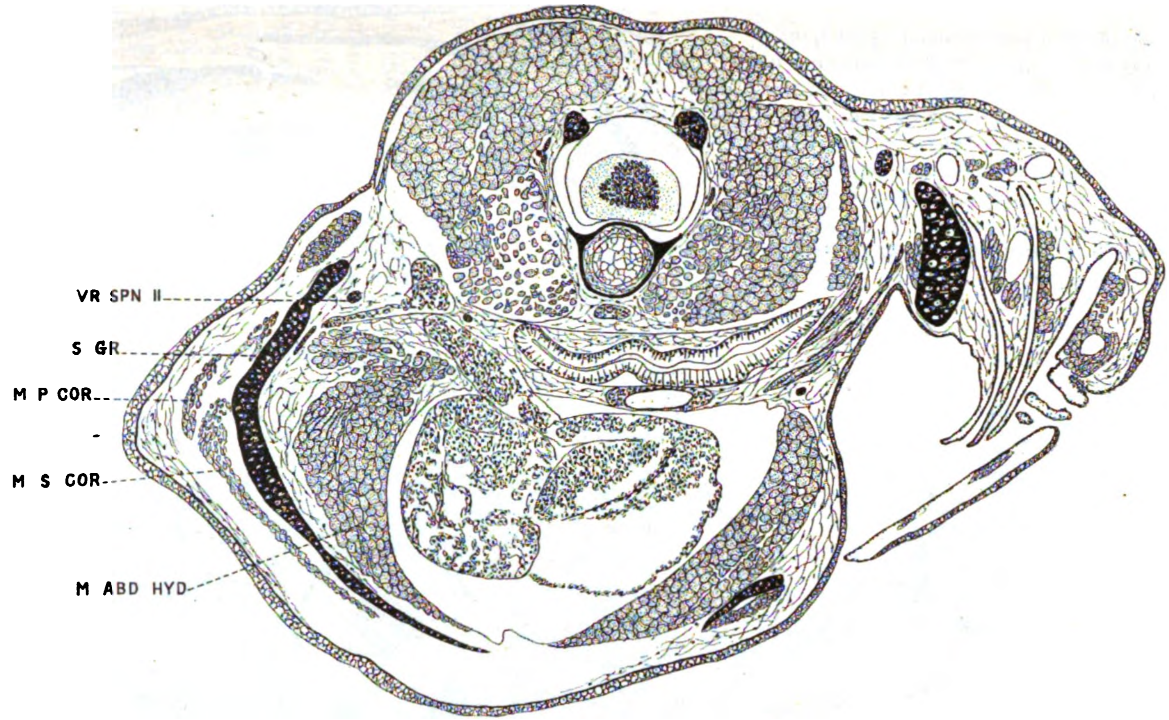

Fig. 31 Drawing of transverse section (no. 470) of case $AA2S_{78}$ at the level *B–B*, figure 17. × 24. *S GR*, shoulder-girdle; *M P COR*, m. procoracohumeralis; *M S COR*, m. supracoracoideus; *M ABD HYD*, m. abdominohyoideus; *VR SP N II*, ventral ramus of second spinal nerve.

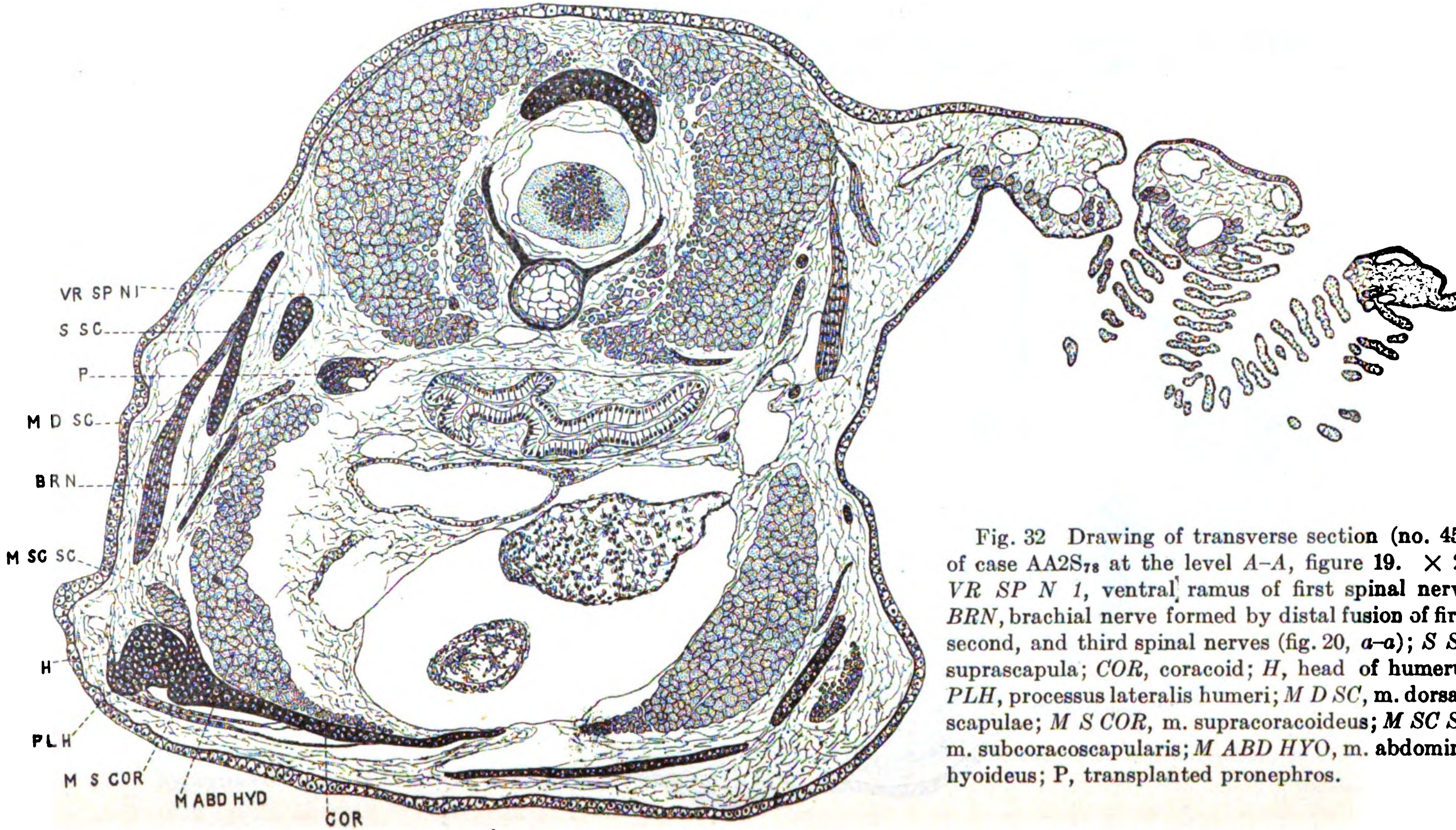

Fig. 32 Drawing of transverse section (no. 455) of case $AA2S_{78}$ at the level *A–A*, figure 19. × 27. *VR SP N 1*, ventral ramus of first spinal nerve; *BRN*, brachial nerve formed by distal fusion of first, second, and third spinal nerves (fig. 20, *a–a*); *S SC*, suprascapula; *COR*, coracoid; *H*, head of humerus; *PLH*, processus lateralis humeri; *M D SC*, m. dorsalis scapulae; *M S COR*, m. supracoracoideus; *M SC SC*, m. subcoracoscapularis; *M ABD HYO*, m. abdomino-hyoideus; P, transplanted pronephros.

Typical gills failed to develop from the tissues lying dorsal to the implanted limb rudiment, the ectoderm migrating out on the dorsomesial surface of the developing appendage. It was in these cases that the free appendage took on the posture of a developing gill (fig. 6). In some cases the limb regained its normal posture by a subsequent ventral rotation, but in the majority of the experiments it underwent abortive development and permanently assumed the pose of a typical external gill (figs. 26 and 29).

That the outgrowth factors for the gills reside not only in the ectoderm of the immediate gill region, but extend with diminishing degree of intensity into the surrounding ectoderm, is evident from these experiments.

The relative gill-producing power of the ectoderm covering specific contiguous outlying regions (e.g., heart, pronephros, etc.) could not be tested in the present work, but it is hoped that this question may form the basis for a future investigation.

Résumé of experiments

Viewing the experiments as a whole, we see that the limbs, although transplanted from one to three segments anterior to the normal position, received in most cases two or more nerves from the original limb level of the cord (table 2). Although the proximal portion of these nerves is seen to assume a normal course, their distal portions have grown in an anterior direction, in spite of mechanical opposition, and have effected functional connection with the transplanted appendage (fig. 16, cf. fig. 4).

The general function of these limbs was found to be less perfect than in limbs transplanted equivalent distances caudal to the normal position (Detwiler, '20, table 1), which also received several nerves from the original limb level of the cord (table 2).

The more incomplete function of the limbs transplanted into the gill region, as compared with that of limbs transplanted the same distance caudal to the normal site, appears to be due to greater structural deficiencies in the girdle and shoulder muscles and to less complete peripheral innervation. The defective

shoulder musculature and imperfect innervation as is seen in table 3 is due no doubt to the active gill-forming properties of the surrounding ectoderm, which in most cases either inhibited the formation of the limb or exerted disturbing influences upon its differentiation. The latter was manifest by considerable torsion in the developing appendage and a high percentage of abnormalities (table 1).

Examination of sections showed that even when the limb was structurally complete, the shoulder, in many cases, was quite distorted. The resultant abnormalities were found to be greater in the musculature than in the girdle itself, which was quite extensively developed in some cases.

Lack of freedom in the motility of the limbs was also due in part to the inhibitory influence exerted by the gill ectoderm which frequently extended out over the proximal portion of the arm (case $AA2S_{25}$, fig. 14). The arm in such cases was not only fused to the side of the body, but the shoulder-joint was deeply situated beneath the surface, thus mechanically preventing freedom in its movements.

DISCUSSION

From the results of the foregoing experiments, in addition to those previously obtained (Detwiler, '20), evidence has accumulated which strongly indicates that there exists between the limb and its normal nerves a developmental relationship which is more intimate in character than any developmental association between these same nerves and other structures. This is suggested by the apparent tendency on the part of the limb nerves to effect functional connection with their proper end organ (limb) when the latter is shifted a given number of segments posterior or anterior to the normal site (table 2 and figs. 5, 8, 16, and 18).

All the previous observational evidence tended to support the idea that the number of segmental nerves contributing to the normal limb plexus is governed by the position and extent of the limb rudiment at the time when it occupied its maximal extent in the embryo, and that plexus formation itself results from

concentration of the rudiment into the definitive limb bud. This is well illustrated in Amblystoma. The original limb rudiment which extends from the anterior border of the third somite to the posterior border of the fifth becomes innervated by the third, fourth, and fifth segmental nerves. Assuming that these nerves reach the rudiment when it occupies its maximal extent, as they very likely do, their convergence into a plexus (fig. 4) would mechanically result from the concentration of the rudiment into the definitive limb bud which centers ventral to the fourth myotome.

In discussing the question of variations in the segmental nerve supply to the limb plexus, Harrison ('10) explained such modifications as due specifically to variations in the position and extent of the limb rudiment, these variations are assumed to occur frequently in nature. Harrison's interpretation of this condition was justly made as a result of his own limb experiments in which he found that transplanted limbs received their nerve supply from segments of the cord corresponding in position to that occupied by the transplanted rudiment. It must be borne in mind, however, that in his experiments, all of which were carried out on anuran forms, the nerve paths were in part or totally laid down at the period when the rudiment was transplanted. In preparing the wound for the reception of the transplant, the peripheral ends of the nerves were severed and the rudiment placed in the direct pathway of the cut ends. Furthermore in these experiments, the normal limb rudiments were left intact in all cases, and the transplanted limb was developed from an additional rudiment taken from another embryo. Under the conditions of his own experiments, therefore, it was impossible to test the effects of variations in the position of the limb upon the growth of its normal nerves, since these nerves were already in connection with their normal intact appendages at the time of the operation.

If the position and extent of the limb rudiment alone determined the source and number of spinal nerves contributing to its plexus, we should expect that the rudiment, when transplanted several segments caudal or anterior to its normal position,

would effect a corresponding shift in its segmental nerve supply. Its failure to do so in these and previous experiments on Amblystoma (table 2) has suggested that other factors must be considered in any attempt to interpret the character of innervation which these cases presented.

The fact that the limb nerves made functional connection with their proper end organ when the latter was transplanted four and five segments caudal to the normal site, scarcely seemed explainable on purely mechanical grounds. These nerves did not terminate at the limbless area as they do upon simple extirpation of the limb, but they continued their growth caudally until the heterotopic limb was reached and connections were made. The caudoventral elongation of the myotomes undoubtedly acts as a mechanical factor in directing the nerves caudally towards the transplanted limb. Such a mechanical influence would not explain why the nerves, being so directed, should make functional connection with the limb after they do reach it any more than mechanical agencies can determine proper selective peripheral connections under normal conditions of development.

It has been shown in previous experiments (Detwiler, '20, '21) that when a limb rudiment is excised, there are, in early stages of development, as many motor axones passing out to the limbless area as there are on the opposite (normal) side. These observations, by the way, strongly contradict those of Shorey ('09), who claimed that no motor axones developed in the absence of the functional end organ. In the absence of the major portion of their terminal musculature (limb and shoulder muscles), the unsupplied fibers in such cases terminated in the general limbless area. However, when the limb was reimplanted further caudally a given number of body segments, the nerves, which in these cases had as many motor axones as are present after simple extirpation of the limb, did not terminate in the limbless area, but continued their growth and effected functional connection with the heterotopic appendage. This observation alone strongly indicated that the extended caudal growth of the nerves in the latter case is not governed solely by the mechanical effects result-

ing from the caudoventral elongating myotomes; otherwise, upon simple extirpation of the limb, the same mechanical influences should be expected to cause the limb nerves to follow a similar pathway.

The intimate developmental relationship between the limb and its normal nerves, as suggested in these experiments, has been more strongly brought out in the experiments in which the limb was transplanted anterior to its normal position. The limb nerves in these cases, in effecting connection with the appendage, have had to do so under the mechanical opposition resulting from the caudoventral growth of the myotomes which tends to direct their growth in a caudal direction. That the nerves in these experiments actually were so directed in their initial outgrowth is seen from the direction of their proximal portions (figs. 8, 13, 16, and 18). The limbs in these cases, although occupying a position from only one to two and a half segments anterior to the normal site, in many cases (table 1 and fig. 3) were originally reimplanted the distance of three segments anterior to the normal site, so that the relative distance between the transplanted limb and the normal limb nerves was greater at the time when initial connections were made than is indicated by the final position of the limb, the latter having migrated caudally during development.

From the examination of figure 3 it is seen that the caudal limit of the transplanted rudiment in series B (table 1) extends only to the anterior level of the second myotome. Under the mechanical influence of the elongating myotomes which direct the nerves caudally, we should hardly expect that the third and fourth nerves would change their initial direction of growth to an anterior course unless there exists in the latter some attractive influence which is responsible for the reversed course of their distal portions (fig. 16).

The orderliness of peripheral growth and terminal connections in normal development offers strong evidence in favor of the presence of some specific reaction between each kind of nerve fiber and the particular structure to be innervated. In discussing the question of peripheral connections, particularly with

reference to selectivity on the part of the sensory and motor fibers, Harrison ('10) suggested an analogy to the union of the sperm and the ovum. Assuming a cell to be in a condition of ripeness, an intimate contact with the nerve fiber would take place and thus terminate the susceptibility of the cell to further innervation, so that other nerve fibers, growing in the same path, would pass along to other cells.

If tropisms do underlie the establishment of the connections between nerve fiber and peripheral cell, there appears to be no reason why such forces should not account for the connections between nerves from a specific region of the central nervous system and a given peripheral system. If such could be shown to be the case, as later experiments may, the unusual character of the nerve plexuses of transplanted limbs in these experiments would meet a ready explanation—particularly those limbs transplanted anterior to the normal situation where connections from the original limb level can only be assumed to take place under the influence of forces stronger than the mechanical opposition which they meet in making their connections.

The tendency on the part of the limb nerves to innervate the displaced limb rudiment is not to be interpreted as a definite specificity between these nerves and their normal muscles, for, in these experiments entire brachial plexuses have been built up from nerves which normally supply the abdominal musculature (Detwiler, '20, series AS5 and AS6). They do suggest, however, that there is a 'preference' on the part of the limb nerves for their normal terminal musculature, otherwise any group of spinal nerves into whose territory a limb is transplanted should effect innervation. That there may be in development such a 'preference' on the part of a group of end cells of the same character (chemical constitution?) for given nerve fibers is suggested in experimental observations on nerve transplantation, in which it has been shown that no nerve can be made to effect functional connection with a muscle unless the normal nervous connection is at first broken (Elsberg, '18). Such a preference as exists here is seen to be different from rigid specificity, since the extraneous nerve can accomplish the same result as the normal

nerve, provided the latter be isolated. The susceptibility of muscle to additional motor innervation is undoubtedly terminated when its initial motor connection is established in development. Such stabilization, however, does not permanently preclude the possibility of a muscle's being functionally innervated by other nerves.

The experiments under consideration in this paper offer no explanation of the nature or character of the stimuli which bring about the proper peripheral connections in the embryo. They do suggest more strongly, perhaps, than have previous observations, the possibility that there does exist in the embryo preferential selectivity at the periphery, possibly of a chemotactic nature, which may largely account for the constancy of connections. Kappers ('17) has offered a rational electrochemical theory in explanation of the dynamic polarization of the neurone and of the selectivity within the central nervous system, and it is possible that peripheral selectivity may be determined in a similar manner.

The initial outgrowth of the peripheral nerve in a straight line and at right angles to the central nervous system as was first observed by His ('88) falls in line with the stimulogenous fibrillation concept of Bok ('15) and meets an explanation on galvanotactic grounds as presented by Kappers (op. cit.). That the nerve fibers in the embryo have only very short distances to grow before connecting with their proper end organs was brought out by Harrison ('10). Thus the principal nerve paths which grow out from the central nervous system are at first relatively short and only later become lengthened out by the shifting of parts which accompanies the development and growth of the organism. Thus we see that, although the final paths of the nerve fiber are purely subsidiary to the growth and shifting of the organ innervated, initial connection cannot be explained on purely mechanical grounds, and it is very probable that there exists, at the periphery, forces of a chemotactic or galvanotactic nature which bring about orderly selectivity just as these same forces appear to underly the process of selectivity within the central nervous system.

SUMMARY

1. Shifting the position of the fore-limb rudiment a given number of body segments anterior or caudal to its normal site, does not effect to the same extent a corresponding shifting of the segmental nerve contribution to the brachial plexus. There is a marked tendency for transplanted limbs to receive innervation from the normal limb level of the cord (table 2 and figs. 4, 5, 16, and 18).

2. The number of segments occupied by a transplanted limb does not determine the number of segmental nerves contributing to its plexus.

3. When the fore-limb rudiment is transplanted from two to three segments anterior to the normal position, the distal portions of the normal limb nerves are found to grow anteriorly the distance of several segments to effect functional connection with the heterotopic appendage. The proximal portions of the nerve follow a normal pathway (fig. 16, cf. fig. 4). In effecting connections with the limb, the nerves apparently grow anteriorly against the mechanical opposition of the developing myotomes, which tends to direct them caudally.

4. From the results of the present limb experiments, in addition to those previously published (Detwiler, '20), evidence has accumulated which strongly suggests that there exists between the limb and its normal nerves a developmental relationship which is more intimate in character than any developmental association between these same nerves and other structures. The contention is thus supported that mechanical influences, although governing in large measure the character of nerve pathways, do not reveal the mechanism by which proper connections are made at the periphery.

5. The function of transplanted limbs is conditioned by four main factors: 1) structural incompleteness of the shoulder-girdle; 2) defective development of the limb and the shoulder muscles; 3) defective peripheral innervation, and, 4) defective connections within the central nervous system.

6. The gradual loss of function in limbs as they are transplanted farther and farther away from the normal situation is

attributed to increased defective connections with the central reflex pathways involved in normal limb locomotion, rather than to a corresponding decrease in effective peripheral innervation and structural deficiencies of the shoulder and limb.

7. The high percentage of abnormalities in limbs transplanted from two to three segments anterior to the normal position (table 1 and figs. 2 and 3) is due to the fact that they are placed in the environment of active gill-forming tissue. In these cases the gill-producing properties of the tissue surrounding the transplant markedly disturb the normal posture and differentiation of the appendage.

8. When the ectoderm and mesoderm are removed from the entire external gill swelling (fig. 3) and a limb is transplanted into the denuded area, abortive gills may develop from the surrounding tissue (table 1). The results of the experiments indicate that the tissue lying ventral to the typical gill region possesses a relatively higher gill-forming capacity than does that lying anterior or dorsal.

9. That the outgrowth factors for the external gills reside not only in the ectoderm of the immediate gill region, but extend with diminishing degree of intensity into the surrounding ectoderm, is evident. These observations, in so far as they could be made in the present work, support the results obtained by Ekman ('13 a, '13 b, '14) in his experiments on anuran forms.

10. The hypoglossal nerve in Amblystoma is formed by the union of the ventral rami of the first and second spinal nerves and corresponds with the arrangement characteristic for urodeles.

11. The entire ventral ramus of the second spinal nerve may contribute nerves to the transplanted limb. In such cases the hypobranchial musculature is supplied by the first spinal nerve.

12. When the ventral portion of the first myotome is excised with the gill mass, the anterior segment of the m. sternohyoideus, the m. geniohyoideus, and the m. hyoglossus are absent on the operated side. The hypoglossal trunk in such cases does not assume its normal anterior pathway, but terminates in the posterior segments of the m. sternohyoideus.

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Resumen por el autor, Harry Lewis Wieman.

Los efectos de la transplantación de una porción del tubo neural de Amblystoma en una posición perpendicular a la normal.

I. Consideraciones generales.

Cuando se extirpa una pequeña sección del tubo neural al nivel del segundo al cuarto somita y se implanta en una posición perpendicular a suposición normal se obtienen los siguientes resultados: 1) El trozo transplantado se desarrolla y retiene su polaridad originaria; 2) Fibras aparecen creciendo posteriormente desde el extremo anterior hacia la pieza transplantada y desde esta hacia el extremo posterior; 3) Las fibras ascendentes crecen hacia delante desde el extremo posterior solamente después de haberse establecido conexiones por medio de las fibras descendentes; 4) La pieza transplantada es reabsorbida en el tubo neural reconstruído, pero hasta cierto punto retiene su polaridad estructural originaria la cual dura hasta cuarenta días después de la operación.

Translation by José F. Nonides
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THE EFFECT OF TRANSPLANTING A PORTION OF THE NEURAL TUBE OF AMBLYSTOMA TO A POSITION AT RIGHT ANGLES TO THE NORMAL

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EIGHTEEN FIGURES

I

It has been known for a long time that when the amphibian spinal cord is severed, in early embryonic stages, restoration of anatomical and physiological continuity takes place (Born, '97; Harrison, '98; Hooker, '15). It is also known that when a section of the cord is excised and replaced in a reverse anteroposterior position, healing per primam occurs if the cut edges are carefully apposed (Harrison, '98, '03; Spemann, '12; Hooker, '15). According to Hooker, the reversed section retains its original morphological polarity, but its functional polarity becomes reversed through adaptation. The nerve fibers show a marked tendency to avoid entering the opposite wound surfaces. The experiments I am about to describe were designed to test further the regulative capacity of the amphibian neural tube.

Eggs of Amblystoma punctatum were collected shortly after deposition and allowed to develop in the laboratory. The stages used for operation extended from the period of the fused neural folds to a point just before the larva becomes sensitive, but most of the operations were made in the earlier stages. The operation consisted in excising a piece of the neural tube and somites, equal to the length of two somites, and replacing it, dorsal side up, at right angles to its original position. Thus the axis of the transplant formed an angle of 90° to the remainder of the neural tube. The length of the transplant was made somewhat longer than the width of the tube, in order to obtain a maximum length

in the transplant, so that reactions and development due to its original polarity could be studied to best advantage.

The operation was accomplished by two transverse incisions through the neural tube and somites, followed by a longitudinal cut on each side connecting the lower ends of the transverse incisions and extending to the midline in a frontal plane. In some cases the notochord was purposely cut and moved with the transplant; in other cases it was left intact. The piece thus freed was lifted out, swung around through 90°, and pressed into the wound. The purpose in removing somite with the neural tube was to preserve as nearly as possible normal conditions for any nerves that might later develop from the transplanted tube, and, at the same time, to provide a non-nervous block between the stumps of the neural tube and the transplant. The usual practice was followed in holding the transpant in place by means of thin glass rods bent to fit snugly over the embryo at the site of operation. From five to fifteen minutes sufficed for the transplant to become attached, after which the holders were removed. After an hour or so in 0.4 per cent salt solution, in which also the operations were performed, the embryos were removed to tap-water and allowed to develop at a temperature of 15° to 20°C.[1]

Following the operation, the embryos were examined from time to time under the binocular, sketched, and tested for nervous conductivity through the transplant by means of delicate tactile stimuli. The material was fixed in corrosive-acetic, embedded in rubber-paraffin, cut into 10μ sections, and stained on the slide. On the whole, the most satisfactory staining results were obtained with Delafield's haematoxylin and orange G.

The stages most frequently used for operations reported on at the present time are shown in figure 1, *A* and *B*; *C* and *D* are outlines of older stages also used. The sketches were made five minutes after the operation. These stages showed no

[1] During the spring of 1920, the author enjoyed the privilege of spending two weeks at the Osborn Zoological Laboratory of Yale University, which afforded opportunity to observe the operative technique as practiced and so highly perfected by Professor Harrison and his students. To Professor Harrison and the zoological staff at Yale the writer begs to express his deep sense of appreciation of the courtesy and privileges extended to him during his visit there.

marked difference in healing, and no constant differences were noted in subsequent changes. The present account is based upon a study of some fifty operations, the results of which were of a uniform character.

Figure 2 illustrates the gross changes in the form of the body typical of embryos operated at stage *D* (fig. 1). Similar changes are noted when the operation is made at earlier stages, except

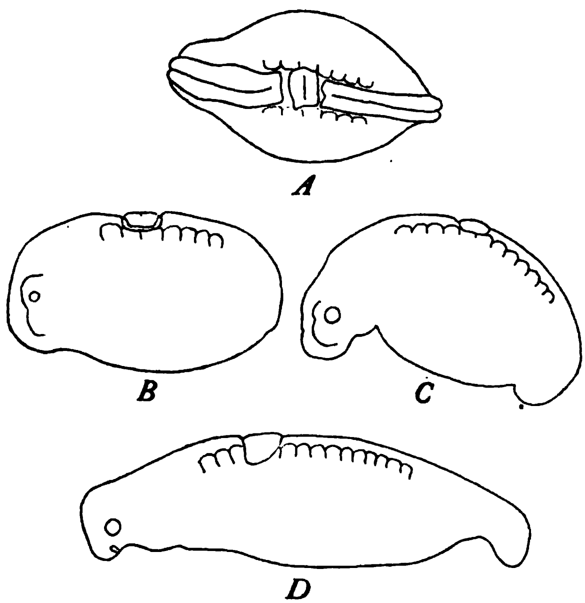


Fig. 1. *A* and *B*, dorsal and lateral views, T. R. and T. L. series; *C* and *D*, lateral views, F and G series, respectively. Five minutes after operation.

that the primary flexing of the head and tail toward each other dorsally, is apt to be more marked when the operating is done in the earlier stages. This dorsal flexure is well shown in figure 3, *A*, and is probably due to contraction accompanying the closure of the wound. As may be noted in figure 2, this preliminary bending is followed by a straightening of the body axis, which in turn is succeeded by bending in a direction opposite to the first bringing the head and tail nearer to each other on the ventral side. It was found that in cases where the ventral bending was

least, or practically absent, the notochord had been cut and moved as a part of the transplant, whereas in all cases where the notochord was left intact marked ventral bending occurred. The principal agents in producing the bent axis are the transplanted neural tube and somites.

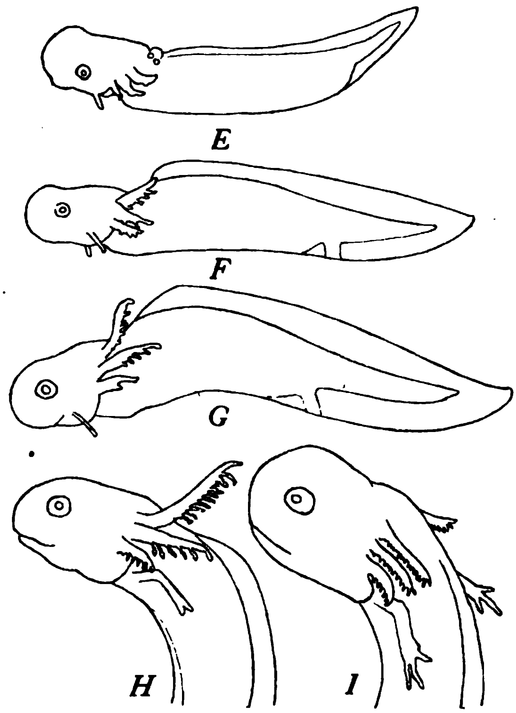


Fig. 2 Embryo G9. Operated April 16. *E*, April 30; *F*, May 4; *G*, May 7; *H*, May 17; *I*, May 26.

Another general effect of the operation was the underdevelopment of the gills, which never became as large and bushy as those of the controls. No special effect was produced on the development of the forelimbs, although many of the operations were made in the region of their nerve supply.

Figure 3, *A*, shows the appearance of embryo T. R. 1, eight days after the operation. In the sagittal section, *B*, the trans-

planted tube can be clearly seen, but as the section passes lateral (left) to the brain and cord of the host, none of the latter can be seen. It is evident that the transplant has progressed in its development, having closed completely and having grown in thickness and length. The section shown in *C* passes nearer to

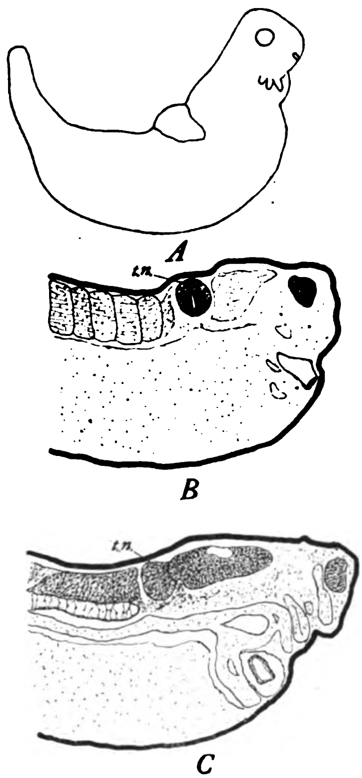
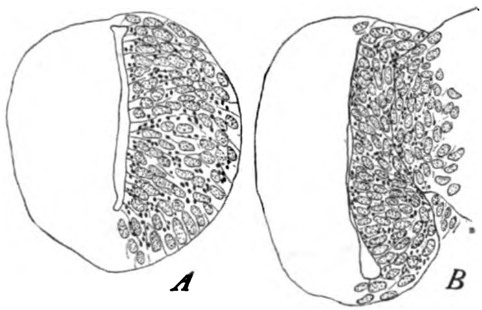


Fig. 3 *A*, embryo T. L. 1; *B* and *C*, sagittal sections. *t.n.*, transplanted neural tube. Eight days after operation.

the midline. It shows the transplant and both stumps of the neural tube with the transplant in close contact with the anterior stump. At first glance, it might appear that these two regions had fused, but a closer examination under higher power (fig. 4) shows that this is not the case, and that the cells of the two regions are rather sharply marked off. The condition was brought

about by the growing brain's extending caudally and denting in the adjacent side of the transplant, without any positive tendency to fusion with the latter. Posteriorly (fig. 3, *C*), the transplant is entirely free of the neural stump; nor is there any evidence in other sections of the series of any connections whatsoever between the posterior stump and the transplant. The



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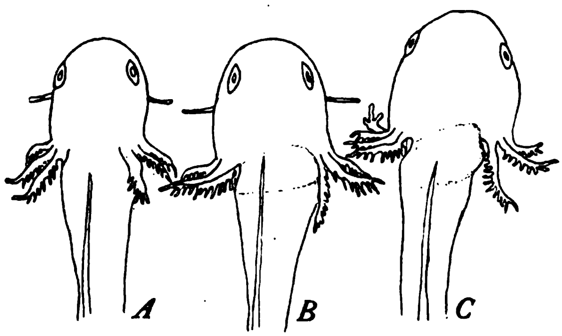

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Fig. 4 *A* and *B*, enlargements of transplanted tube of figure 3, *A* and *B*, respectively.

Fig. 5 Embryo T.R.3, operated April 8. *A*, April 28; *B*, May 3; *C*, May 10.

relation shown in figure 4, *B*, may represent a first step in the restoration of anatomical continuity between the anterior stump and the transplant, but since it is also true that in other embryos of this series and age the transplant is entirely free of contact with either stump, it would seem that the condition found in T. R. 1 is more or less the result of a chance contact brought about by the rapid growth at this time of the brain stem anterior

to the transplant. There is much reason to believe that definitive union does not take place until actual nerve fibers develop.

Figure 5 and figure 6, *B*, show sketches of embryo T. R. 3, belonging to the same series as the preceding. Twenty days after the operation (*A*), there is scarcely any visible outward evidence of the operation except lessened size as compared with

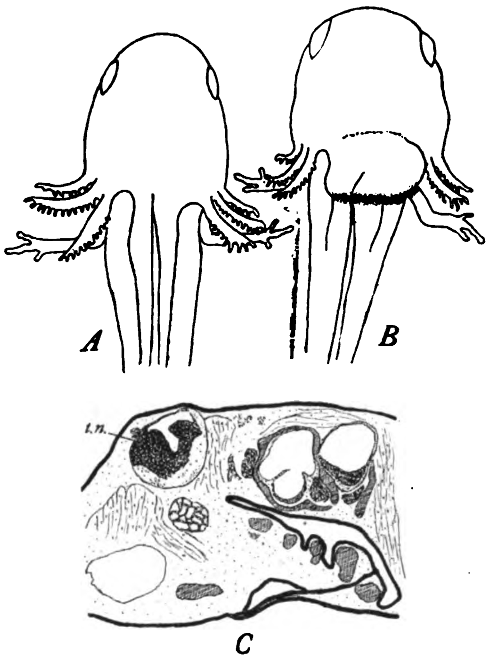


Fig. 6 Embryo T.R.3. *B*, May 17; *C*, sagittal section. *A*, control. *t.n.*, transplanted neural tube. In this and all subsequent drawings of sections the shaded parts represent cartilage.

the control. Five days later (*B*), a slight transverse swelling appeared at the site of operation, which became more marked a week later (*C*). Finally (fig. 6, *B*), thirty-seven days after the operation, the transverse ridge became well defined and projected slightly beyond the line of the body on the right. At this point the animal was killed and fixed. In this embryo there was practically no ventral bending of the body; the animal maintained an upright position and was very active. Its body length was

somewhat less than that of the control. The notochord was cut and the section moved with the transplant. Tactile stimulation in front of and behind the transplant gave no evidence of nervous conduction in an anteroposterior, or reverse, direction.

The production of a transverse ridge was not a constant feature of operated embryos. The fact is mentioned because it happened to be present in this particular embryo which was one of those selected for sectioning. Sections show that the position of the ridge does not coincide exactly with the position of the transplanted tube. The ridge was found to be due in part to the growth in length and breadth of the transplant, but principally to growth and differentiation of transplanted somites especially toward the right side (fig. 6, *B*). This larger projecting end lies near the original posterior end of the transplanted neural tube.

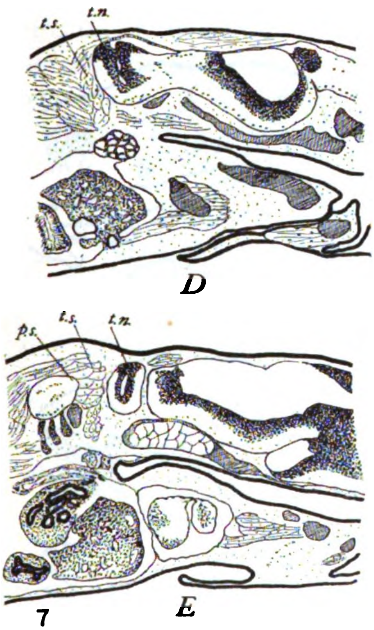
Figure 6, *C*, shows a section in a sagittal plane passing through the eye, otic vesicle, and the enlarged (original anterior) end of the transplant. The sections begin at the animal's left side. The appearance of the section shows that the transplant has not only increased in size, but has undergone differentiation typical of the hindbrain region from which it came.

A section 180μ nearer to the midline (fig. 7, *D*) and to the right shows a very broad connection between the transplant and the brain in front. The section shown in *E* passes 100μ to the right of *D*. The transplant is free, having no connections on either side. The posterior stump is seen in this figure with three thick nerve processes passing ventrally. It is separated from the transplant by muscle derived from transplanted somite.

Figure 8, *F*, 140μ to the right of *E*, passes approximately through the midline, and shows the transplant free and underdeveloped. The amount of development to be expected is indicated by the appearance of the posterior stump, which is large, showing every indication of differentiation. It terminates in a smooth, rounded surface.

The section seen at *G* is 120μ to the right of *F* and shows both stumps with the transplant underdeveloped and lying free between them.

The sections give an idea of the conditions at various levels as seen in selected sagittal sections. They show all of the important points that have come to light from the complete study of the entire series of sections of this embryo and others of the same age. These points may be summarized as follows: 1) Evidence of an active growth and differentiation of the original



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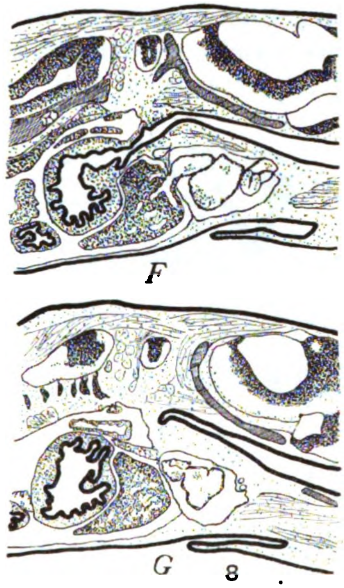

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Fig. 7 Embryo T.R.3, sagittal sections. *t.n.*, transplanted neural tube; *t.s.*, transplanted somite; *p.s.*, posterior stump.

Fig. 8 Embryo T.R.3, sagittal sections.

anterior end of the transplanted neural tube; 2) the presence of a well-defined connection between the transplant and the anterior stump located to the left of the midline, that is, toward the original anterior end of the transplant; 3) the absence of any connection between the transplant and the posterior stump; 4) evidence of active growth and differentiation in the posterior stump; 5) evidence of progressive atrophy in the transplant to the right of its union with the brain, that is, in the direction of its original posterior end.

Embryo T. R. 5 (fig. 9) developed a marked ventral bend in the body which interfered with its maintaining an upright position. The notochord was not disturbed by the operation, which was performed April 8th. Tactile stimulation from time to time showed no evidence of conductivity anteroposteriorly, or reverse, through the transplant. Thus for May 10th my notes show the following record:

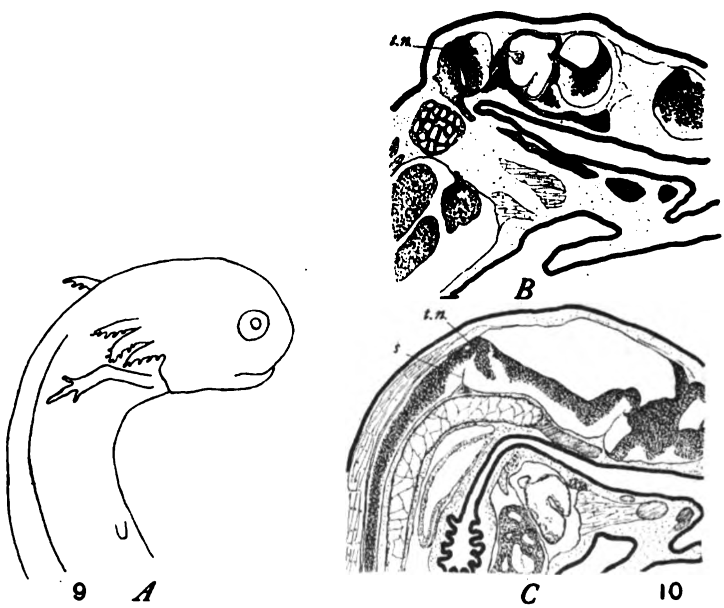


Fig. 9 Embryo T.R.5, May 17. Operated April 8.
Fig. 10 Embryo T.R.5, sagittal sections. *s*, connective-tissue septum.

Stimulation anterior to transplant: slight twitching of gills.

Stimulation posterior to transplant: active body movement; head quiet.

Stimulation at site of transplant: active twitching of gills.

This record points to a descending connection from the anterior stump to the transplant, but no posterior connection. In the course of a week the character of the responses changed. Thus on May 17th the record is as follows:

Stimulation anterior to the transplant: active movement of forelimbs, followed by quick body contractions.

Stimulation posterior to transplant: similar response.

Stimulation at site of transplant: similar response.

These results indicated that conduction was occurring back and forth through the transplant—that functional connections had been established between the transplant and the two stumps.

Figure 10, *B*, shows the original anterior end of the transplant in close contact with the ganglia of the ninth and tenth nerves, with which a union seems to have been formed. A large process passes ventrally from the transplant just in front of the pro-

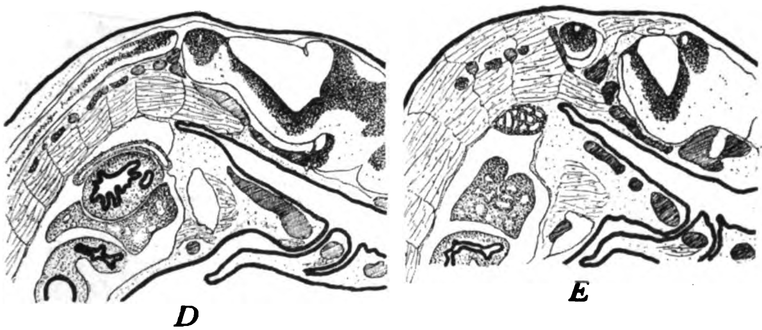


Fig. 11 Embryo T.R.5, sagittal sections

nephros. The section shown in *C*, passing through the midline, shows the transplant completely incorporated in the nervous system. The irregularity in the distribution of the nuclei and the connective tissue septum (*s*) indicate the position of the transplant. The fact that anatomical continuity is established leads to the belief that the results of stimulation noted above were due to nervous conductivity through the transplant rather than to direct muscular stimulation.

Figure 11, *D*, is a section passing to the right of the midline. It shows a well-defined union between the transplant and the brain in front. Section *E* passes still further to the right. The transplant shows signs of diminished vigor and growth, and is not connected with either stump.

Figure 12 is an outline of embryo T. R. 4, an animal belonging to the same series as the preceding, but not killed until May 27th. For some time preceding this date responses to stimulation indicated that conduction paths had been established through the transplanted tube.

In figure 13, *B*, is seen a sagittal section passing to the left of the midline. Complete anatomical continuity is evident, but

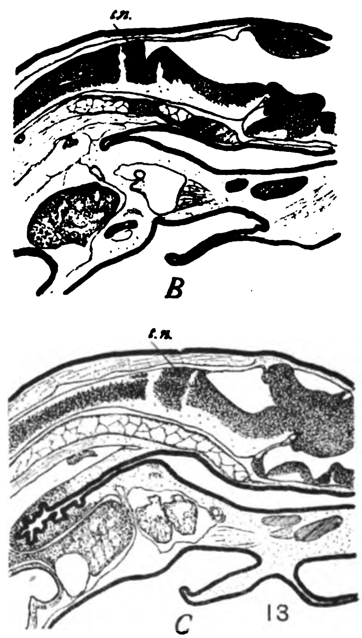


Fig. 12 Embryo T.R.4, May 27. Operated April 8
Fig. 13 Embryo T.R.4, sagittal sections

the V-shaped configuration of the nuclei reveals the location of the transplant. The section shown in *C* passes directly through the midline, where the transplant appears to be completely absorbed; but here again its boundaries are indicated by two breaks or gaps in the nuclear mass. Careful examination of all of the sections in the series shows that the fusion between the transplant and the nervous system is very intimate, none of the transplant extending beyond the lateral limits of the brain and cord.

A study of transverse sections was also made, but a description of such sections would be merely a repetition of what has already been described. For this reason but one transverse series will be considered here, and that because it throws some light on the completeness with which anatomical union is established through the transplant.

Embryo T. R. 7, shown in the figure with the control, had a history similar to that of T. R. 4. Like the latter it showed normal responses to stimuli twenty days after the operation, was very active and, save for a slight bend in the posterior part of its body and its slightly smaller size, behaved very much like

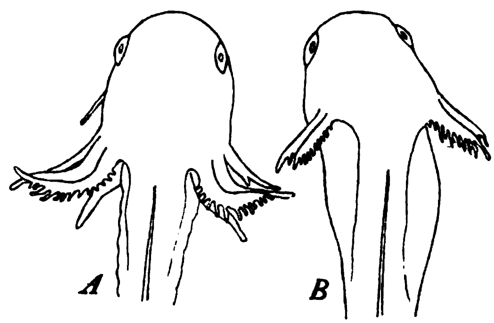


Fig. 14 *B*, Embryo T.R.7, May 5. Operated April 8. *A*, control.

the control. It was killed May 25th, twenty-five days after the operation, and by that time its body had straightened out considerably.

Transverse sections showed complete fusion of the transplant, but its location could be determined by the configuration of the cells and the irregularity in the shape and position of the canal as well as by the appearance of the regenerating notochord, which in this case had been cut. (It was left intact in T. R. 4.) Figure 15, *C*, shows a transverse section passing just in front of the transplant. *D* passes through the region of contact between the transplant and the anterior stump. It is at once evident that the cells are abnormally distributed and that there is no evidence of a canal. *E* passes through the center of the transplant. It shows a misplaced canal, and also the remains of the transplanted

notochord at *t. no.* Regeneration in the notochord is taking place in the stumps; the transplanted piece appears atrophied and seems to take no part in the process. *F*, passes through the posterior limit of the transplant. Here again the canal is missing. The section also shows the overlapping ends of the regenerating notochord. Posterior to this level the sections are normal.

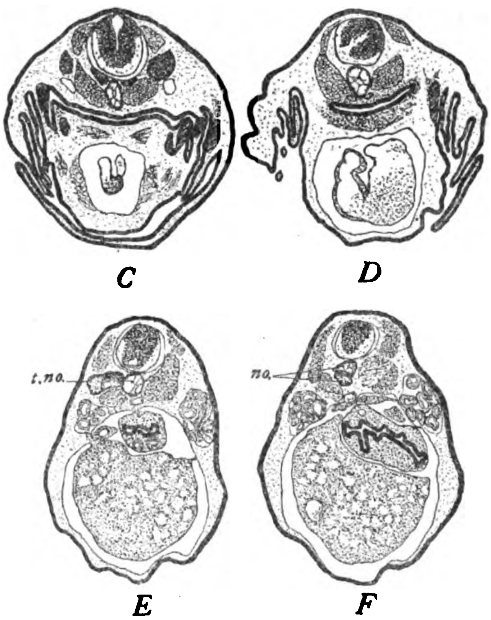


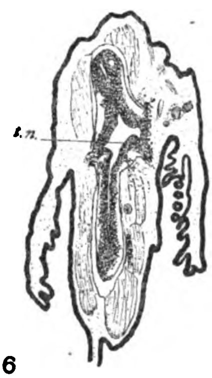
Fig. 15 Embryo T.R.7, transverse sections. *t.no.*, transplanted notochord; *no.*, regenerating ends of the notochord.

Therefore, the conditions found in the transverse series confirm what has already been shown in the sagittal series of T. R. 4.

Frontal sections afford a view of the process from still a different angle and supply further confirmation of the conclusions already reached. Embryo G9 was cut into frontal sections for this purpose. It is shown at the operating stage in figure 1, *D*. Figure 2 consists of drawings of the embryos at various intervals up to the time of killing, May 17th. Responses to tactile stimulation indicated that conduction pathways were established

through the transplant by May 7th (fig. 2, *G*), seven days after the operation.

The frontal section shows the transplant united with both stumps, but the readjustment is not as complete as in the two preceding cases, which was to have been expected from the shorter duration of time between operation and fixing in the case of G9. In the figure the original anterior end of the transplant is to the right. Owing to a shift in position, its axis cuts that of



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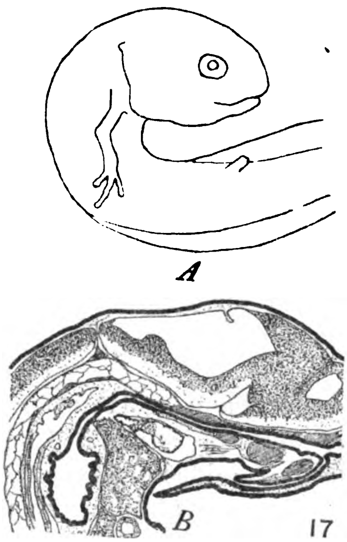

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Fig. 16 Embryo G9, frontal section. Anterior end up.
Fig. 17 *A*, Embryo T.L.9, May 26. Operated April 8. *B*, sagittal section.

the embryo diagonally, the anterior end of the transplant lying cephalad to its posterior end. The shift forward of the anterior end of the transplant might have been due to factors connected with the process of bridging the stumps, or it might have been due to lack of success in implanting the excised tube at right angles to the embryonic axis at the time of operating. However, the fact that a similar shift in position of the transplanted tube has been noted in many other operated embryos points to the conclusion that it is not the result of accident in technique, but rather an indication of a definite orientation of the transplant.

It is also to be noted in figure 16 that the original anterior end of the transplant (to the right) is larger than the opposite end, and that the axis of the brain stem (above) points toward this larger end. Thus it would seem that the junction at this point with the transplant was brought about by descending fibers growing back from the brain not in a straight line, but toward the original anterior end of the transplant. This same fact appears from an examination of sagittal sections, where it may be recalled that the connection between the anterior stump and the transplant lies for the most part to one side of the midline, the side nearer to the original anterior end of the transplant.

Figure 16 also shows that the connection with the posterior stump leaves the transplant from its posterior end (to the left), which would be expected on the assumption that the transplant had retained its original anteroposterior polarization. As a result there is a zig-zag in the restored neural axis, which, evidence from older stages (figs. 12 to 14) shows, may be later partially straightened out.

II

The disturbance caused by the operation to which these embryos were subjected must be profound, and it seems remarkable that reëstablishment of morphological and functional continuity to the extent noted occurs with such frequency in the relatively small number of cases that I have studied. However, the success of the operation depends to some extent upon the region in which the transplantation is made. Thus in the experiments considered up to this point the transplantations were made in the region of the second to the fourth somites approximately. Among these experiments only one case occurred from which recovery seemed impossible or at least unlikely.

Embryo T. L. 9, operated April 8th, showed by its reactions to stimulation that connections through the transplant were established by April 28th. At this time the embryo showed a slight bend (ventral) in the body axis. As time went on, the bend became more pronounced and response to stimuli more and more sluggish, until by May 26th, when it was fixed, no evidence

could be obtained of conduction through the transplant. By this time the bend in the axis had become extremely acute (fig. 17, *A*).

Sections showed that the loss in conductivity was due to the formation of a cartilaginous partition cutting through the neural axis just behind the transplant. Figure 17, *B*, a section in the medial plane, passes through the thinnest part of the partition and shows the transplant firmly united in front with the brain. In all probability the transplant completed its anterior connection, but was unsuccessful in developing a posterior connection of sufficient stability to resist the pressure of the growing posterior wall of the brain capsule, with the result that a foramen magnum failed to develop, and the brain became completely hemmed off anteriorly.

III

In the F series of embryos the operation was made in the region of the fifth and sixth somites, and the results of these experiments are somewhat different from those of the other series. All were operated the same day, April 16th, and none of them during thirty days following the operation showed any evidence, by the method of tactile stimulation, of nervous continuity through the transplant. The operating stage is shown in figure 1, *C*.

Let us consider a few examples. F7, killed May 7th, twenty-one days after the operation, showed in sections that the transplanted tube was widely separated from the stumps of the brain and cord by muscle that had developed from transplanted somites. The transplanted tube had, however, undergone a certain amount of growth and differentiation, and showed no signs of atrophy. Practically the same conditions were found in F1, killed May 17th, ten days later than F7. F3, killed May 18th, one day after F1, displayed in sections the same wide separation between the transplant and the stumps, but in this case the transplant gave every appearance of atrophy in its reduced size and ragged form. Had the embryo been allowed to live, the possibility of its establishing connections between the

neural stumps would seem to have been very remote indeed. Finally, F4, killed May 17th, was the only one of the entire series that in sections showed any connection between transplant and nervous system, and here the connection extended only between the anterior side of the transplant and the brain. The appearance of the sections does not exclude the possibility of a posterior connection through the transplant being established

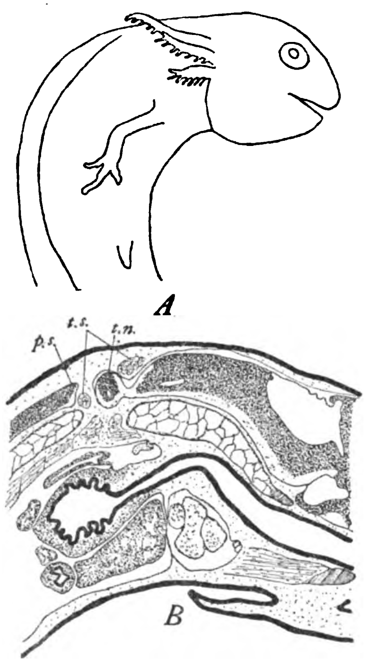

Fig. 18 *A*, Embryo F4, May 17. Operated April 16. *B*, sagittal section.

later had the embryo been allowed to live. However, the fact that this had not occurred by the end of thirty-one days speaks against it, since in the T. R. series both anterior and posterior connections were established as early as twenty days after the operation, and in the G series even earlier—seven days in the case of G9—but the latter series were much older at the time of operation (fig. 1). Further, F4 was the only one of the six embryos of the F series chosen at random for sectioning in which

there was any indication of any connection between the transplant and the stumps.

Only twelve operations were made in the F series, and this number is of course too small to serve as the basis for far-reaching conclusions; nevertheless, the fact remains that the results in this series differed consistently from the others in that restoration of anatomical and functional continuity was delayed or even excluded. At the time the operations were performed it was not forseen that a matter of a few somites difference in level would make a material difference in the reaction of the neural tube to the operation; but it can be pointed out now that the results obtained are what one should expect if the initial step in the restoration process is a posterior growth of fibers from the brain to the transplanted tube, since the longer time required for fibers to reach the more posterior level would allow somites intervening between transplanted tube and the neural stumps to develop to such an extent as to delay materially, if not actually to prevent, the formation of a nervous connection. Therefore the nearer to the brain the transplantation is made the better the chances for connections being established. This point will be subjected to further experimental test, the results of which will be reported upon later.

IV

We may now review in their proper order the various steps involved in the process of restoring anatomical and functional continuity between the transplanted tube and the rest of the nervous system. In the first place, at the time of the operation in the T. R, T. L., and F series, no tracts were established, so that there was nothing to interfere with localized development taking its natural course; and as a result neuroblasts develop in all parts of the neural tube, including the transplanted portion. Since a stage just before appearance of sensitivity was used for operation in the G series, the first steps in the formation of the primitive somatic sensory and motor tracts had already started in this series. In the second place, whenever in the sectioned embryo a single connection was found between the transplant

and the neural stumps, the connection always occurred between the anterior stump and the transplant. Since an outgrowth fails to appear at this time from the posterior side of the transplanted tube, there is little reason to suppose that such an outgrowth takes place from its anterior side either; for which reason it would seem that the anterior connection is initiated by descending fibers from the anterior stump.

It is not known to what extent, if any, nerves developing from the transplant participate in this process; but since a certain amount of axial mesoderm was left attached to either side of the transplanted tube, it would seem that such nerves would develop their usual relations with somite and skin (Coghill, '14), rather than deviate from their ordinary path of development, which would still be open to them, to develop unusual relations with the anterior stump.

The rapid growth of the brain pushing the anterior stump against the transplant may be an incidental factor in bringing about a union, but complete fusion does not occur until fibers develop. This is illustrated by the results of the G series, in which it was found that connections between transplant and stumps developed in a much shorter time after operation than in the other series. In other words, the formation of a connection between the stump and transplant depends upon the state of development of nerve processes. The location of the connection between the anterior stump and transplant, as seen in figure 18, *B*, clearly indicates its motor character.

As regards the connection between the transplant and the posterior stump, everything points to its being initiated by the continued growth backward, through and beyond the transplant of descending fibers from the anterior stump, augmented perhaps by similar fibers from the transplant itself. In the first place, the participation in this process of nerves arising from the transplant may be ruled out for reasons already given. In the second place, there is no indication of processes growing forward from the posterior stump even after well developed anterior connections are formed (fig. 7). While the posterior stump at this time shows every indication of growth and development, its surface

is smooth and rounded without the slightest trace of outgrowth forward. In fact, the appearance of sections points to what amounts to a repulsive effect, or negative chemotaxis, between the posterior stump and transplant, or at least the complete absence of any attraction between the two regions. The significance, therefore, of the time interval between the formation of the anterior and posterior connections would seem to be that it represents the time required for the descending fibers from the anterior stump to make their way through the transplant; which they do in the direction of the original anteroposterior axis of the transplant. After traversing the transplant a short distance, the descending fibers again change their direction and leave the transplant to bridge the gap between it and the posterior stump. All evidence of repulsion between the two regions would then disappear just as soon as the descending fibers have opportunity to penetrate the posterior stump, which they do, presumably, because the posterior stump is territory that they traverse in normal development.

Incidentally, if the above interpretation be the correct one, it indicates that the first long tracts connecting the brain and cord are motor. After the motor connections are established through the transplant, there is every reason to believe that sensory tracts develop, with the result that there is restored a condition that is almost normal, but not completely so, for the period during which the embryos were under observation. To what extent the restoration approaches normal conditions remains to be seen.

Hooker ('17), in his study of the effect of reversing a section of the cord in the frog, found a marked tendency on the part of the nerve fibers to avoid entering the opposite wound surfaces. The conditions of his experiments excluded any opportunity for the dissipation of this state of mutual repulsion, with the result that nerve connections failed to develop between the ends of the reversed cord and the stumps. In my experiments the conditions are different, and the 'avoiding reaction' is more likely a phenomenon of the posterior stump only—the transplant being neutral, so to speak. The question as to why ascending fibers do not arise from the posterior stump would seem to have its answer

in the results of the experiment, namely, the development of ascending tracts from the cord to the brain awaits and therefore depends upon the formation of motor tracts. Once the stumps have been bridged by descending fibers, all semblance of repulsion between the posterior stump and the transplant disappears, and the way is prepared for ascending tracts to grow forward.

Hooker ('15), after severing the cord of the larval frog in the cervical region, found that when the wound surfaces are not apposed the first steps leading to a reunion and return to nearly normal form and structure are brought about by, 1) the development of nerve fibers from the motor cells of each segment of the cord and, 2) the growth of sensory axones from the cut surface of the posterior stump. In other words, the motor connections precede the sensory. Coghill ('13), in his study of the primary ventral roots and the somatic motor column of Amblystoma, has shown that the ventral root fibers occur in their full relation between the spinal cord and muscle some time before the muscle can be stimulated through the sensory field. The physiological properties can in no sense be determined through stimulation in the sensory field, for they may be actually functional for some time before they come under the influence of the sensory nerves. Thus Coghill's work indicates that in the formation of the early reflex arcs in the cord the motor connections are established before the sensory. My results similarly point to the formation of the long motor tracts from the brain to the cord before the sensory tracts.

It is also to be noted that the restoration of nervous connections through the transplant takes place in the direction of the normal metabolic gradient of the embryo. Such a gradient is also exhibited in the transplanted tube, the original anterior end invariably exhibiting evidence of more vigorous growth and developmental energy than the posterior end. This is true even after a connection has formed between the anterior stump and the transplant. The fact that this connection is always made at a point nearer to the anterior end of the transplant might explain the difference in behavior of the two ends of the transplant, were it not for the fact that a similar difference in the two

ends exists in cases where the stumps have failed to form unions with the transplant (F series). At the same time it seems doubtful that the transplant can for an indefinite period maintain its form and structure without establishing connections with the stumps of the brain and cord. This is indicated by the results of the F series, in which the operation was made at the level of the fifth and sixth somites, and in which in only one case out of four selected for sectioning was even a single connection developed between the stumps and the transplant within a period of thirty days. In the other three cases the transplant was separated from the neural stumps by muscle tissue to such an extent that nervous union seemed unlikely, if not impossible. In two of these the transplant showed evidence of growth and development, the original anterior end being larger, but in the third (F3) the transplant was disintegrating. Presumably, the degeneration of the transplant was due to its failure to form a connection with the nervous system.

As has already been mentioned, the results of the F series supply additional evidence for the conclusion reached regarding the manner in which the nervous stumps become reunited through the transplant. In the T. R, T. L., and G series the operation was performed in the region of the second to fourth somites, while in the F series the operation was behind the fifth somite. If the restoration of anatomical connection depends upon the downgrowth of descending fibers from the brain, then a longer time would be required for such fibers to reach the site of operation in the F series. The results obtained in the F series would seem, then, to be due to the fact that the greater amount of time required for the descending fibers to reach the level of operation allowed transplanted somite to develop to such an extent on either side of the transplanted tube as to form a barrier on either side of it. That this barrier may be overcome on the anterior side of the transplant, at least, in the course of thirty days is shown by F4 (fig. 18), but whether or not similar connections would have been made later on in all of the F series is of little moment to the point under discussion. The primary reason for lack of successful reunions in the F series would seem to be the more posterior site of the operation as compared with the other series.

V

General conclusions may be summarized as follows:

I. When a small section of the neural tube of Amblystoma, at the stage of the closed neural folds, is removed at the level of the second to fourth somites, together with portions of adjacent somites, and reimplanted at right angles to its normal position:

1. The transplanted tube continues its development and retains its original polarity.

2. The growing brain, pushing the anterior stump back against the anterior side of the transplanted tube, may be a factor in forming a union at the point of contact.

3. Nerve fibers grow back from the anterior stump into the transplant near its original anterior end.

4. The posterior stump becomes club-shaped, but shows no initial tendency to send fibers forward into the transplant.

5. A connection between the transplant and the posterior stump is brought about by the continued growth backward of fibers from the anterior stump through the transplant.

6. Ascending fibers then grow forward from the posterior stump.

7. The transplant eventually becomes absorbed in the reconstructed neural tube, but the appearance of sections shows that the cells of the transplant do not lose their original polarity as late as thirty to forty days after operation.

II. When the operation is performed at a stage just before the larva becomes sensitive, the process is the same, but the union is formed in a shorter time after operation.

III. When the operation is performed at the earlier stages, but in the region of the fifth and sixth somites, or farther back:

1. A longer time is required for the formation of nervous connections.

2. In series of twelve operations no case of complete connections between the stumps occurred in thirty days.

3. In one case at least the transplant was disintegrated at the end of thirty days.

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Resumen por los autores, Lorande L. Woodruff y H. Spencer.

Estudios sobre Spathidium spatula.

I. La estructura y comportamiento de Spathidium con especial mención de la captura e ingestión de su presa.

Los puntos esenciales consignados en el presente trabajo son: 1. Spathidium no posee tricocistos y por consiguiente la extrusión de tales estructuras no es la causa de la parálisis de la presa. 2. Existen triquites en la región oral formando una empalizada que es en apariencia comparable a los bastones de soporte que rodean la boca en los más generalizados ciliados gimnostómidos. No existe prueba positiva de que los triquites sean el punto donde se origina el veneno. 3. Spathidium spatula en los cultivos de los autores posee micronúcleos claramente definidos. Por esta causa el Spathidium estudiado por Moody (Journ. Morph., '12) debe interpretarse como una raza amicronucleada. 4. El alimento de Spathidium se limita a pequeños ciliados, pero no a miembros del género Colpidium. 5. Spathidium se pone en contracto con su presa solamente al azar. 6. Una presa que ha sido paralizada y se ha desprendido de la región oral de Spathidium puede ser recobrada en la mayor parte de los casos mediante una serie compleja de reacciones sucesivamente modificadas indicadoras de la existencia de una "sensibilidad a distancia." 7. El factor que juega un papel en esta sensibilidad a distancia es aparentemente una substancia segregada por Spathidium cuando la presa es paralizada.

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STUDIES ON SPATHIDIUM SPATHULA

I. THE STRUCTURE AND BEHAVIOR OF SPATHIDIUM, WITH SPECIAL REFERENCE TO THE CAPTURE AND INGESTION OF ITS PREY

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EIGHT TEXT FIGURES AND ONE PLATE (FIGURES NINE TO TWENTY)

The holotrichous infusorian which today bears the name Spathidium spathula apparently was first described by Müller in 1773, and again in 1786 as "Enchelis cylindrica, apice hyalina spathulata" and designated Enchelis spathula.[1] The next observations of importance were those of Ehrenberg[2] in 1830 and 1838, which led him to place the organism in the genus Leucophrys as L. spathula. In 1841 Dujardin[3] founded the genus Spathidium for an organism which he considered identical with Müller's Enchelis spathula, and named it Spathidium hyalinum. Eighteen years later, in his monograph on the Infusoria, Stein[4] merely mentioned the organism incidentally, and apparently was of the opinion that it was not specifically distinct from Ehrenberg's Enchelys farcimen, and retained it in the genus Enchelys. Maupas[5] in 1888 recognized the genus Spathidium of Dujardin and the species spathula of Müller, and thus established the modern name of the organism, Spathidium spathula. The most recent work on the structure and the first on the

[1] O. F. Müller, Vermium terrestrium et fluviatilium historia, 1773, p. 38. Animalcula Infusoria fluviatilia et marina, 1786, p. 40, pl. V, fig. 19.

[2] C. G. Ehrenberg, Abhandl. der Akad. d. Wissensch. zu Berlin, 1830, S. 42; 1831, S. 105. Die Infusionsthierchen als volkommene Organismen, 1838, S. 312.

[3] F. Dujardin, Histoire naturelle des Zoophytes. Infusoires, 1841, p. 458.

[4] F. Stein, Der Organismus der Infusionsthiere, 1859, I. Abtheilung, S. 80.

[5] E. Maupas, Sur la multiplication des Infusoires ciliés, Arch. de Zool. Exp. et Gen., 1888, (2) 4, p. 246.

life-history of Spathidium was done by Moody[6] in 1912, and her study is accordingly the point of departure for the discussion of our own observations.

MATERIAL AND METHODS

The original animal of the pedigree cultures which we are conducting was isolated on November 5, 1920, from a jar of decaying vegetable debris collected at New Haven. Up to the present time a large number of separate lines have been started from the original one, some in which conjugation has been prevented and others in which it has been allowed to occur.

The various pedigreed lines have been carried on depression slides kept in large moist chambers. The culture medium, which was supplied daily at the time of isolation, consisted of two drops of standard beef extract[7] plus a drop of hay infusion which had previously been seeded with small ciliates, chiefly Colpidium. The bacteria which developed afforded food for the small ciliates and these in turn provided food for the Spathidia.

The normal living animals, specimens stained intra vitam, preserved total preparations, and serial sections have been studied both with the monocular and binocular compound microscope with various lens systems, including the Zeiss 2-mm. apochromat, and compensating oculars 4 to 18. The Zeiss binocular dissecting microscope, with lens combinations affording magnifications up to 172 diameters, has also been employed.

For fixation, Schaudinn's solution (two parts saturated solution of corrosive sublimate and one part of absolute alcohol) proved most satisfactory for general cytological studies. In special cases osmic-acid vapor, Zenker's fluid, corrosive-acetic, Worcester's fluid, picric acid, and various other fixatives were utilized. A wide range of stains was used, many of them intra vitam. We may mention Lyon's blue, methylene blue, methyl

[6] J. E. Moody, Observations on the life history of two rare ciliates, Spathidium spathula and Actinobolus radians. Jour. Morph., 1912, vol. 23, p. 349.

[7] L. L. Woodruff and G. A. Baitsell, The reproduction of Paramecium aurelia in a 'constant' culture medium of beef extract. Jour. Exp. Zoöl., 1911, vol. 11, p. 135.

green, nigrosin, dahlia, Bensley's mitochrondria, gentian violet, Loeffler's, Mallory's triple stain, picro carmine, Delafield's haematoxylin, and Heidenhain's iron haematoxylin.

MORPHOLOGY OF SPATHIDIUM

The general structure of Spathidium as described by Moody is corroborated by the study of the animals of our pedigree cultures. The flask-shaped form of a typical vegetative individual is well illustrated in figure 9. But specimens immediately before and after division, encystment, and conjugation, as well as when starved and after a heavy meal, exhibit very marked changes from the typical condition. In addition to the diversities in the relative proportions of the various parts of the cell under these varying circumstances the contortions of the organism, when it becomes entangled in zoogloeal debris, etc., augment the protean picture which it presents, though we have never seen a specimen 'double its length' as recorded by Moody. But such a succession of forms is apparent that many might be—indeed, some have been—designated as distinct species (Cf. figs. 9, 10, 13, 14, 19, and 20). As a matter of fact, the attempt to determine the specificity of widely different specimens in a wild culture led to the discovery of the peculiar advantages afforded by Spathidium for experimental purposes.

Cilia. The ciliation of the specimens in our cultures agrees with Moody's description, there being about sixteen longitudinal rows of cilia evenly distributed over the cell. However, each of these bands is in turn made up of two closely opposed rows of cilia. It has been impossible to demonstrate this composite character of the bands in living specimens, but sections clearly reveal a double row of basal granules in each band. At the edge of the truncated anterior end there are two rows of somewhat longer and stouter cilia, the stronger beat of which is partially responsible for the characteristic gyrations of the anterior end as the organism swims forward revolving on its long axis.

The cytostome consists of a long narrow depression extending almost, if not quite, the whole length of the truncated end nearly at right angles with the long axis of the cell. At the bottom of

the depression there is a slit-like opening into the endoplasm. It is impossible to determine the limits of the opening except when the animal is starting to feed, since at other times the edges are closely appressed. However, when a small ciliate is seized, it rapidly opens and the prey is forced in by means of the contractile edges of the cytostome, assisted apparently, in the earliest stages, by the activity of the larger cilia in this region.

Trichocysts. Maupas and Moody describe a row of minute rod-like bodies near the edge of the peristome. Maupas gives no details in regard to these bodies, apparently taking it for granted that they are trichocysts. But Moody definitely states that "Three artificial methods were used to explode the trichocysts: exposure to osmic acid vapor, treatment with a 2 per cent solution of acetic acid and a solution of methyl green. After treatment with any one of these reagents, in fifty individuals examined, the cilia were found freely extended, the trichocysts being readily distinguished from the long oral cilia inasmuch as they were straight and stiff in appearance, whereas the cilia showed a wavy outline." This statement seems to be sufficiently positive to settle the question, though farther on in the same paragraph the author states that she has not "found the greatly elongated trichocysts of complicated structure described by Maier, Schuberg, and Schewiakoff for Frontonia, Paramecium and other ciliates; owing, however, to the paralyzing effect on Colpidium following contact with the anterior end of the body, I think it safe to interpret, as trichocysts, the short opaque rods so plainly visible in the thickened rim of the mouth."[8]

We have been unable to demonstrate the extrusion of trichocysts in our material, although we have tried all the methods which are effective in other ciliates, such as Paramecium, including the reagents used by Moody herself. In favorable specimens the basal granules of the oral cilia can be seen and present somewhat the appearance of 'trichocysts' as shown in Moody's figure 12. However, they are identical in structure with the basal granules of the smaller cilia on the general surface

[8] Loc. cit., p. 356.

of the cell. Accordingly, since living animals, studied under the highest powers and manipulated with the Barber micro-dissecting apparatus, stained total mounts, and serial sections fail to reveal the presence of trichocysts, we are forced to conclude that they are not present.[9]

Although trichocysts were discovered by Ellis[10] as long ago as 1769, given their present name by Allman[11] in 1855, and supposed to be 'poison organs' by Lachmann[12] two years later, there is to-day little conclusive data in regard to their function. Even in forms like Paramecium and Frontonia with obviously highly developed trichocyst apparatus, so far as we are aware, the function of the trichocysts is chiefly a matter of assumption. Mast clearly showed that the trichocysts of neither of these organisms have any paralyzing effect on Didinium, and attributed to them merely a mechanical effect in that they form a viscid mass about the organism which hampers its enemy. Indeed, Jennings stated: "It is possible that the discharge (of trichocysts) is really an expression of injury,—a purely secondary, even pathological phenomenon, like the formation of vesicles on the surface of an injured specimen."[13]

On the authority of Balbiani,[14] trichocysts were stated to occur in Didinium until Thon,[15] corroborated by Mast,[16] demonstrated their absence. However, Thon believed that Didinium has a paralyzing effect on a Paramecium which has been struck by the

[9] L. L. Woodruff and Hope Spencer, The food reactions of the infusorian Spathidium spathula. Proc. Soc. Exp. Biol. and Med., 1921, vol. 18, p. 183.

[10] J. Ellis, Philosophical Trans. Royal Society, 1769, vol. 59, p. 144.

[11] G. J. Allman, On the occurrence among the Infusoria of peculiar organs resembling thread-cells. Quart. Jour. Microscopical Science, 1855, vol. 3, p. 177.

[12] J. Lachmann, Annals and Magazine of Natural History, 1857, vol. 19, p. 126.

[13] H. S. Jennings, Behavior of the lower organisms, 1906, p. 91.

[14] E. G. Balbiani, Observations sur le Didinium nasutum. Arch. d. Zool. Exper. et Gen., 1873, T. 2, p. 363.

[15] K. Thon, Ueber den feineren Bau von Didinium nasutum. Archiv f. Protistenk., 1905, p. 289.

[16] S. O. Mast, The reactions of Didinium nasutum (Stein) with special reference to the feeding habits and the function of trichocysts. Biol. Bull., 1909, vol. 16, p. 91.

seizing organ. Calkins[17] apparently showed that there is such an effect, and in the absence of trichocysts interpreted certain deeply staining 'granular striae' near the apex of the trichites comprising the seizing organ as representing the seat of the poison. Actinobolus radians also may be mentioned; both Erlanger and Calkins[18] held that this ciliate captures Halteria grandinella by means of trichocyst-tipped tentacles. But Moody's study of Actinobolus reduced the 'trichocysts' to 'dark granular trichocyst material.'[19]

There is nothing comparable in structure nor, so far as has been proved, in function between trichocysts, in the original sense of the term, and the 'offensive trichocysts' described by Maupas, the 'needleform trichocysts' of other authors, or 'trichocyst material' of still others. Although in the present state of knowledge the line of demarcation is ill defined in many cases between bodies which are purely supporting in function, others which possibly are both supporting and offensive or defensive, and lastly true trichocysts, only confusion is served by extending the significance of the word trichocyst, which in sensu strictu has a very definite connotation, to widely divergent structures.[20]

Trichites. Although trichocysts are not present in Spathidium, there are slender rodlets imbedded like a paling in the thickened rim of the anterior end of the cell which give it a striated appearance under certain conditions. These apparently are what Moody (fig. 18) interprets as parallel thickenings of the cortex which reinforce the pharynx. As a matter of fact, the rodlets may be identified as trichites, provided the use of this term does not necessarily imply that they are the seat of the poison. In addition to the trichites in the oral region, it is possible to identify individual trichites distributed in the endoplasm of the cell. Some

[17] G. N. Calkins, Didinium nasutum: I. The life history, Jour. Exp. Zoöl., 1915, vol. 19, p. 225.

[18] G. N. Calkins, The Protozoa, 1901, p. 50.

[19] Loc. cit., p. 372.

[20] E. Maupas, Contribution à l'Étude Morphologique et Anatomique des Infusoires Ciliés. Arch. d. Zool. Exper. et Gen., 1883, (2), 1, p. 611. Y. Delage and E. Hérouard, La Cellule et les Protozoaires, Paris, 1896, p. 434.

of these apparently are newly formed and being transported to the oral region, while others may well be trichites which have been torn away during the process of prey ingestion (fig. 12).

In view of the fact that this organism paralyzes its prey, it is natural to seize upon the chief structural differentiation of the oral region as the locus of the poison. But on account of the problematical function of clearly defined trichocysts when present, and the absence of any proof that the trichites of Spathidium are actually the seat of the poison, we prefer not to prejudge the question. Though, it is true, Blochmann in particular has interpreted the trichites of certain other ciliates as related to poison production. The trichites of Spathidium are apparently the 'Tastkörperchen,' identified by Stein in an organism which probably is a Spathidium, and the 'trichocysts' noted by Maupas.

The trichites of Spathidium obviously are comparable to the rods which form a framework for the mouth in many of the more generalized gymnostomatous ciliates and undoubtedly are homologous with the strands comprising the highly specialized seizing organ of Didinium. Thon believed that in Didinium he could discriminate between trichites and 'Stäbchen des Reusenapparates,' but no such distinction is possible in Spathidium. The paling of trichites suggests that the whole surface of the truncated anterior end of Spathidium is homologous with the cytostome of such a form as Enchelyodon. In other words, the mouth proper of Spathidium is really the depressed area bounded by the slightly thickened edges of the anterior end of the animal. It is always open and leads to the slit-like cytopharynx immediately below, the sides of which remain appressed except when food is being ingested. This interpretation also seems to be supported by the method by which food is engulfed, which involves a grasping and 'mouthing' of the quarry by the region bearing the trichites.

Contractile vacuole. The Spathidia of the cultures under consideration characteristically possess one large contractile vacuole situated at the posterior end of the cell, though under certain undetermined conditions animals now and then appear with two smaller, closely opposed contractile vacuoles in the same situation.

But sooner or later the two merge into one large vacuole. In this regard our animals are exactly similar to those in Moody's cultures.

Nuclei. The nuclear apparatus of Spathidium as exhibited by our animals is remarkably variable, though it agrees, in the main, with the description given by Maupas. The typical vegetative cell apparently contains two long ribbon-like macronuclei which extend nearly the whole length of the cell (fig. 11). However, owing to the intertwining of these bands, we have been unable to decide whether they are actually distinct or whether they are united at the posterior end to form a single structure. It seems probable that both conditions occur because the macronucleus varies considerably from day to day; very many otherwise typical animals showing the macronucleus fragmented into a few or many separate pieces. Thus far it has not been possible to associate these morphological macronuclear changes with characteristic events in the life-history.

Maupas states that there are from six to nine micronuclei present in Spathidium and the same is apparently true for the organisms of our culture. Seven are visible in the animal illustrated in figure 11. In the majority of cases they are difficult or impossible to identify owing to the fragmented condition of the macronuclear apparatus and the presence of remnants of the nuclei of ingested Protozoa.

Moody was unable to discover any micronuclei in the animals of her culture and states: "Although it is generally thought that the possession of two types of nuclei is characteristic of the ciliates, the present observations give no evidence of this differentiation in Spathidium."[21] In this connection it is important to note that Moody says: "Conjugation was not observed, though numerous attempts were made to bring it about." From the standpoint of the micronucleus, then, Moody's animals were characteristically different from those of our cultures, which not only are micronucleate, but the micronuclei function in a typical manner during conjugation. Indeed, the chief value of the animals of this culture is the ease with which they may

[21] G. N. Calkins, The Protozoa, 1901, p. 358.

be induced to conjugate.[22] Moody's amicronucleate race is undoubtedly comparable with the amicronucleate races of various ciliates recently described by Dawson,[23] Landis,[24] Woodruff,[25] and Patten.[26]

FEEDING BEHAVIOR

Spathidium is one of the so-called hunter ciliates, employing for food the smaller ciliates which it captures during its perigrinations. Maupas, in his study on the life-history of Infusoria, carried for a short time a culture of this organism which he fed chiefly on Glaucoma, Cyclidium, and Cryptochilum. Moody found it impossible to conduct her long pedigree culture without Colpidium for food and definitely states that she never saw any other ciliates paralyzed or eaten. The animals of our culture readily paralyze and swallow almost any small ciliate with which they come in contact, though flagellates of various kinds are immune. As a matter of fact, however, we have employed chiefly species of Colpidium for food because they can be developed for the purpose in countless numbers so readily in small flasks of hay infusion.

Spathidium typically swims forward quite rapidly through the water, revolving on its long axis, and gives at first glance the impression that it is exploring its path with its truncated anterior end. This appearance results chiefly from the fact that the axis of revolution does not pass directly from posterior to anterior end, but leaves the body a short distance posterior to the mouth region. Accordingly, the narrower anterior end traces the circumference of a circle of greater diameter than the rest of the body, especially when the animal is swimming

[22] L. L. Woodruff and Hope Spencer, The early effects of conjugation on the division rate of Spathidium spathula. Proc. Soc. for Exper. Biol. and Medicine, 1921, 18. The survival value of conjugation in the life history of Spathidium spathula. Ibid, 1921, 18.

[23] J. A. Dawson, An experimental study of an amicronucleate Oxytricha. I, Jour. Exp. Zoöl., 1919, vol. 29, p. 473. II, ibid., 1920, vol. 30, p. 129.

[24] E. M. Landis, Amer. Naturalist, 1920, vol. 54, p. 453.

[25] L. L. Woodruff, Jour. Exp. Zoöl., 1921, vol. 34, p. 329.

[26] M. W. Patten, Proc. Soc. for Exper. Biology and Medicine, 1921, vol. 18, p. 188.

rapidly. In addition, the narrow anterior end is quite flexible, and this makes the behavior still more complex.

A careful analysis of the reactions of Spathidium to various stimuli reveals quite clearly that in general its behavior can be interpreted in accordance with the 'avoiding reaction' as worked out for various Protozoa by Jennings. In most cases when a Spathidium, which is swimming forward, is stimulated, it reverses the effective stroke of the cilia, moves back for a short distance, turns toward the side with the shorter anterior tip, and then proceeds forward again on a slightly different course. So far its behavior appears quite stereotyped. But not so when the anterior end strikes a small ciliate. Spathidium now exhibits a series of reactions which may well be fundamentally of the same nature as its others, since Spathidium is limited, of course, by the means at its command, but no good purpose will be served by attempting to interpret it in the same terms.

A casual survey of a number of hungry Spathidia swimming among Colpidia gives one the impression that the Spathidia chase their prey as one tiny ciliate after another falls victim to the carnivores—one of the most spectacular sights in the world of the Infusoria. But a careful study soon makes it clear that the captures are the result of the chance contact of the oral region of a Spathidium with a small ciliate. Indeed, when attempting to follow the movements of a single Spathidium through a heavy culture of Colpidia in order to study the details of the swallowing process, one is frequently astounded to find how easily they miss their prey! Nothing happens unless the quarry strikes the anterior end of the Spathidium nearly or quite in the center. Touching the edges produces no effect on either animal.

But when a Colpidium or similar ciliate is fairly hit, the picture changes instantly with respect to both of the animals involved. The prey, which up to this moment was swimming rapidly, usually instantly becomes motionless and quickly shows pathological changes involving vacuolization of the cytoplasm and disintegration of the cilia. Sometimes, however, paralysis and death are not quite so rapid, and under these conditions the quarry appears to tremble and moves haltingly away for a short

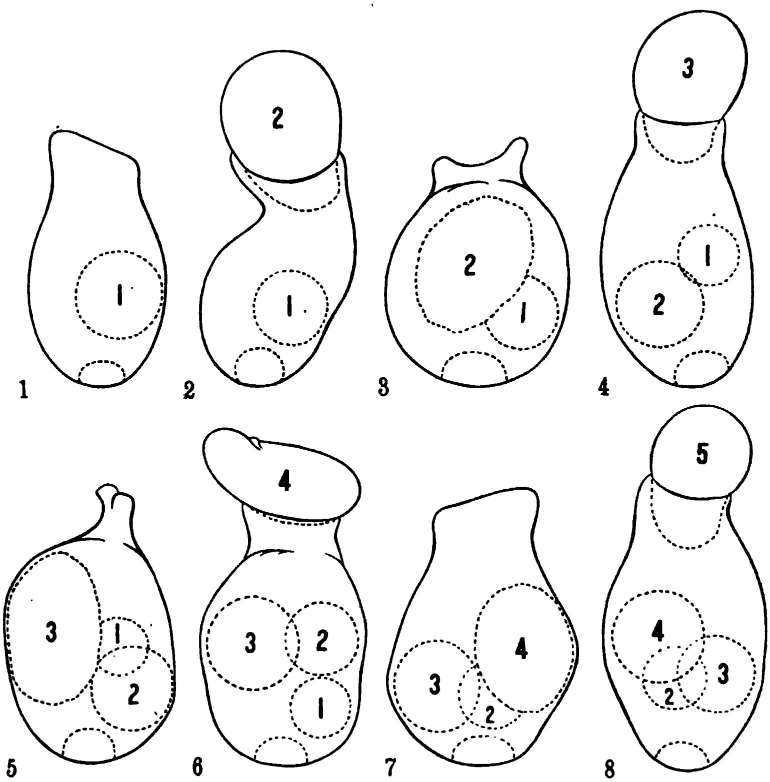
distance. The behavior of Spathidium differs markedly under these two conditions.

If the prey is instantly paralyzed it frequently remains against the oral region of its captor and the reactions of the latter are then relatively simple. The stimulus afforded by the contact immediately stops the forward movements of the Spathidium, which gives a few rapidly repeated avoiding reactions that tend to keep it at or near the spot where the capture was made. Meanwhile, the prey is moved around, apparently as a result of the combined activity of the longer oral cilia and the gradually expanding edges of the truncated end of the Spathidium, until it may conveniently be encircled. This process of mouthing successfully accomplished, nothing further is visible except the gradual sinking of the prey through the greatly expanded cytostome of the captor into the endoplasm (figs. 17, 18, and 19). From the point of ingestion the course of the food vacuole within the cell may be readily followed, especially when the prey has previously been fed with powdered carmine (cf. figs. 1 to 8). The whole process from contact to complete ingestion takes place in about thirty seconds, and the Spathidium, which usually sinks to the bottom during the process, within a few seconds more resumes its active foraging. Three such captures have been observed to occur within eight minutes. Even certain conditions which must be considered as highly abnormal apparently interfere not at all with food taking. For example, a Spathidium transfixed to a cover-glass with the needle of a Barber microdissection apparatus will paralyze a Colpidium and swallow it, provided, of course, the latter remains in contact with the mouth of the Spathidium.

Although the behavior of Spathidium is characteristic and remarkable when it makes a direct capture, the most interesting and significant cases are those in which either the prey is not instantly paralyzed, so that it moves a short distance away, or, though rendered motionless, becomes removed from the oral region of the Spathidium. Under these circumstances, the factors involved obviously become highly complex and the behavior of the Spathidium is not identical in any two cases. The study

of a large number of such captures, however, justifies, we believe, the following general description.

When the prey fails to remain in contact with the oral region, the Spathidium immediately gives a series of avoiding reactions, which, unlike those that occur when the prey remains in contact, tend to increase in extent so that the organism not only remains in the general region in which the attack occurred, but



Figs. 1 to 8. Food-taking by a single specimen of Spathidium spathula. 1. Animal when first observed. Contains one food vacuole enclosing a Colpidium (*1*). The contractile vacuole is shown at the posterior end of the Spathidium in all the figures. 2. Seizing a second (*2*) Colpidium. 3. Second Colpidium enclosed in food vacuole. 4. Third Colpidium being engulfed. 5. Third Colpidium in food vacuole. 6. Seizure of fourth Colpidium. 7. Fourth Colpidium within vacuole; food vacuole with first Colpidium no longer discernible. 8. Engulfing of fifth Colpidium. Observations, extending over about an hour, and drawings by Miss J. Elizabeth Lovett, artist to the Osborn Zoological Laboratory.

also moves backward and forward in the neighborhood of its prey. It is obvious, of course, that such reactions sooner or later will again bring the mouth of the Spathidium in contact with the food. But, a careful analysis of many cases clearly shows that usually these stereotyped reactions are insufficient to account for the comparative precision exhibited by the Spathidium in recovering its quarry. And further, the reactions of the Spathidium are successively modified so that, although it may have become removed several times its own length, within a relatively short time the oral region again comes in direct contact with the food, which is then engulfed in the manner previously described. This is a fact that we believe is indisputable. Of course, such a result as the above does not invariably occur; sometimes the prey is never found again. But, when all the complex factors involved are appreciated, it is remarkable how few paralyzed ciliates are lost.

A Colpidium before being paralyzed has no effect whatsoever on Spathidium; after paralysis it appears to be a center of attraction, and not only to the Spathidium whose oral region it touched, but also in a much less marked though significant degree to other Spathidia in the immediate neighborhood. This last observation naturally suggests the probable explanation. Chemical substances excreted by the dying organism, or secreted at the time of contact by the captor itself, afford a stimulus which results in the characteristic behavior of Spathidium. It is probable that the effective substance in question is secreted by the Spathidium itself because the oral region of the latter is frequently, especially after an unsuccessful capture, a stimulus to neighboring specimens. And the same factor may well be responsible for the peristomal fusion incident to conjugation. Whatever the explanation may be, the point not to be lost sight of is that Spathidium 'senses at a distance' its paralyzed prey.

The reaction against the early anthropomorphic interpretations of the behavior of the Infusoria resulted in a conception of the simpler animals which almost left out of account the fact that they are organisms. The study of Spathidium adds one more to the body of data being rapidly brought to the fore that makes

it increasingly difficult to interpret Protozoan behavior in simple terms.

SUMMARY

The essential points presented are:

1. Spathidium does not possess trichocysts, and consequently the extrusion of such structures is not responsible for the paralysis of the prey.

2. Trichites are present in the oral region, and form a paling which is apparently comparable with the supporting rods about the mouth in the more generalized gymnostomatous ciliates. There is no conclusive evidence that the trichites are the locus of the poison.

3. Spathidium spathula, in our cultures, possesses clearly defined micronuclei. Therefore, the Spathidium studied by Moody must be interpreted as an amicronucleate race.

4. The food of Spathidium is limited to small ciliates, but not to members of the genus Colpidium.

5. Spathidium comes in contact with its prey solely through chance.

6. Prey which has been paralyzed and has become removed from the oral region of the Spathidium is recovered in a majority of instances by a complex series of successively modified reactions, indicating 'sensing at a distance.'

7. The factor involved in the 'sensing at a distance' is apparently a substance secreted by the Spathidium when the prey is paralyzed.

May 6, 1921.

PLATE

PLATE 1

EXPLANATION OF FIGURES

9 Typical foraging Spathidium.

10 A common form when food is scarce.

11 Optical section of total mount, stained with Delafield's haematoxylin, to show macronuclear and micronuclear apparatus. Seven micronuclei are present, one at the posterior end above the contractile vacuole.

12 Section (4μ) to illustrate the anterior paling of trichites and the trichites distributed through the endoplasm; nuclear apparatus, etc., omitted. Heidenhain's iron haematoxylin.

13 Form of Spathidium in an early stage preparatory to encystment.

14 Form shortly before encystment.

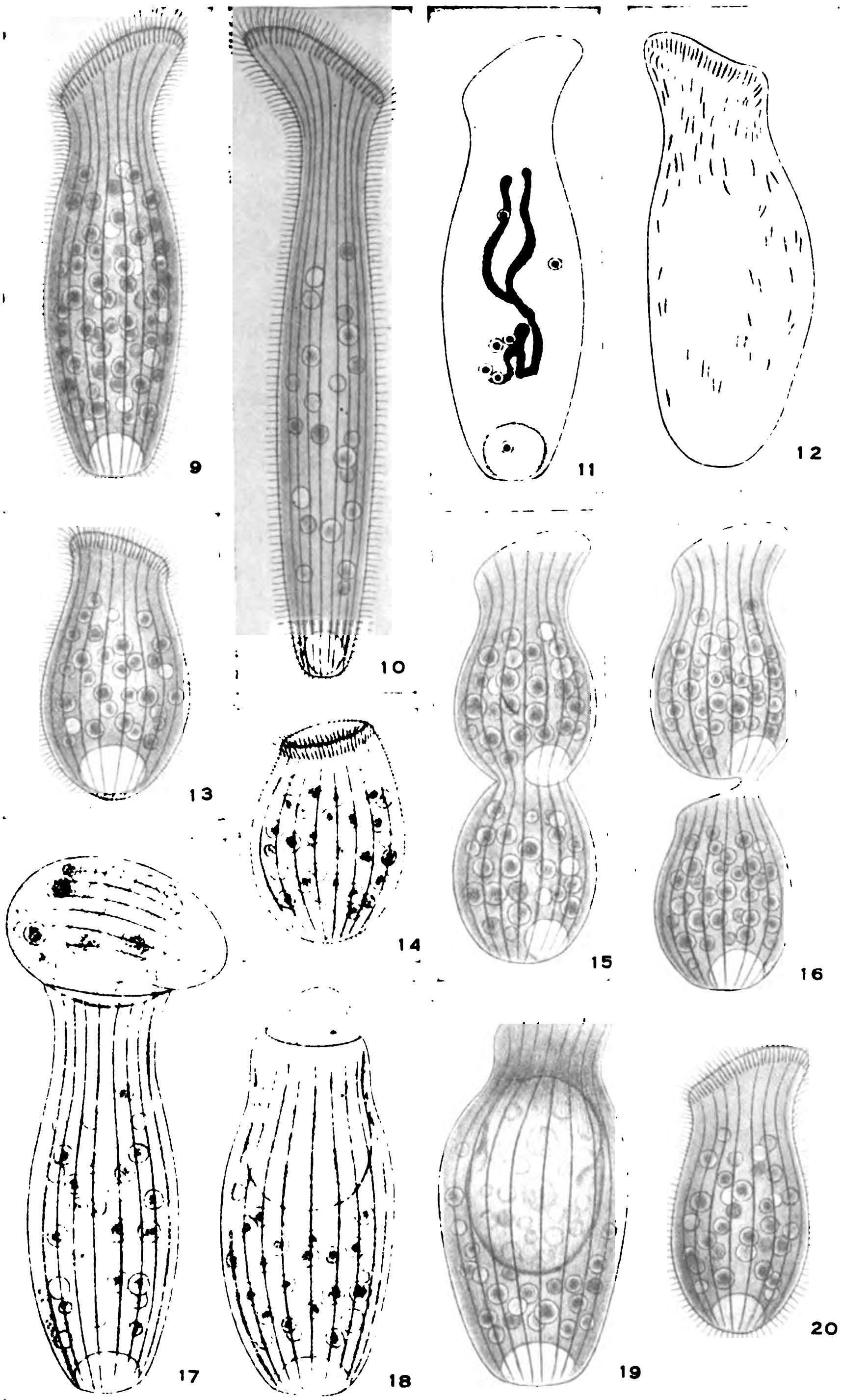
15 Spathidium dividing.

16 Division nearly completed.

17 to 19 Spathidium swallowing a Colpidium. Successive stages.

20 Form shortly after division or when quieting down preparatory to encystment.

Drawn by Miss J. E. Lovett.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Resumen por el autor, M. F. Guyer.

Estudios sobre las citolisinas.

III. Experimentos con las espermatotoxinas.

Las gallinas sujetas a inyecciones repetidas de espermatozoides de conejo producen un suero espermatotóxico violentamente tóxico para los espermatozoides de conejo in vitro, y tóxico en varios grados para los espermatozoides in vivo. Como efecto extremo dicho suero tóxico puede inducir completa esterilidad y degeneración de los túbulos seminíferos. Las espermatotoxinas tóxicas para los conejos son también tóxicas para los espermatozoides del conejillo de Indias in vitro. Los conejos pueden formar espermatotoxinas contra sus propios espermatozoides. Del mismo modo los conejos machos inyectados intravenosamente con espermatozoides de conejo presentan sus espermatozoides debilitados. Un conejo macho formará anticuerpos contra sus propios espermatozoides cuando estos son introducidos en su sangre. El hecho de que un animal puede formar anticuerpos contra sus propios tejidos cuando estos se desplazan o sufren lesiones puede ser importante bajo el punto de vista de la herencia.

Translation by José F. Nonidez
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STUDIES ON CYTOLYSINS

III. EXPERIMENTS WITH SPERMATOTOXINS

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Zoölogical Laboratory, University of Wisconsin

ONE FIGURE

After it had become established that the blood-serum of a given animal acquires a marked solvent action for the red blood corpuscles of another species if the red cells of the latter are injected repeatedly into the animal in question, similar experiments were tried with other kinds of cells. It soon became established that lytic or toxic sera could be developed against such substances as leucocytes, nervous tissue, and spermatozoa. As long ago as 1899[1] specific spermatolysins or spermatotoxins were produced which rapidly killed or at least immobilized the spermatozoa of the species of animal used as the source of the antigen. These early experiments were apparently all made on spermatozoa in vitro, not in the living animal. Later, however, De Lester reported having rendered male mice sterile for from sixteen to twenty days by the injection of such spermatotoxic serum.

To one interested in cytological and genetical problems it becomes a matter of considerable importance to discover just what can be done toward affecting spermatozoa in vivo, and if they can be so affected, to determine how far back in their career the effect is observable. That is, does it modify only the adult spermatozoa, or does it influence also the spermatids, or possibly the spermatocytes?

[1] Metchnikoff. Ann. Past., XIII, 1899. Von Dungern. Münch. med. Woch., S. 1228, 1899.

Again, from experiments of Guyer and Smith[2] with crystalline lens, it appears that antibodies for rabbit lens when introduced into the blood-stream of pregnant rabbits may occasionally attack the lens tissue of young in utero and also, directly or indirectly, specifically affect the germinal correlatives of this tissue in the germ-cells of such young. It is of great importance from the standpoint of heredity, therefore, to determine whether or not an animal can build antibodies against its own tissues when these become misplaced, altered, or injured in some way. If such auto-antibodies may on occasion be constructed, then the way seems clear to a reasonable inference of how certain types of germinal changes are induced. Because of their distinctive nature and the ease with which they may be isolated, spermatozoa lend themselves admirably to such a test.

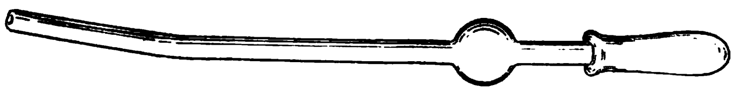

Figure 1

With these thoughts in mind, the experiments which follow were undertaken. The spermatozoa of rabbits were used as the source of the antibodies. The treated males were tested from time to time, both by breeding experiments and by direct microscopical examination of their semen. To secure sufficient semen, a male was allowed to copulate two or three times with a female and the semen was drawn off from the latter by a pipette of a type devised by members of the Department of Genetics. The instrument (fig. 1) has a length of 18 cm., and is made of glass tubing, 6 mm. in diameter.

RABBIT SPERMATOTOXIN IN FOWL-SERUM

Experiment 1

The first test was made with two males, one untagged and the other numbered 4A7. The three fowls used for sensitization were injected intraperitoneally on January 22nd, 29th, and

[2] Jour. Exp. Zoöl., vol. 26, no. 1, May, 1918; ibid., vol. 31, no. 2, August, 1920.

February 5th, 14th, and 20th, respectively, in 1920, with the semen derived each time from three male rabbits, after the semen had had sufficient normal salt solution added to it to make a total quantity of 6 cc. This made a dosage of 2 cc. per fowl. Treatment of the two males with the sensitized fowl-serum was begun on March 6th. Both were first examined to see that their spermatozoa were normal in quantity and activity. The untagged male was given the following quantities of this serum on the dates indicated: March 6th, 3 cc. plus 1 cc. Locke's solution; March 8th, 3 cc.; March 11th, 4 cc.; March 13th, 4.5 cc.; March 15th, 4 cc. On March 3rd, when he was tested, his spermatozoa seemed normal in quantity and activity, and a female to which he was bred on March 13th bore six young. He had become very emaciated during later treatment and died March 18th before further tests could be made.

The dosage and dates of treatment of male 4A7 with the sensitized fowl-serum were as follows: March 6th, 3 cc. plus 1 cc. Locke's solution; March 8th, 3 cc.; March 11th, 4 cc.; March 13th, 6 cc.; March 15th, 4 cc. After the first injection (March 6th) he was mated in order to clear out all spermatozoa that might be in ducts outside the testes. On March 18th, three days after the fifth injection of sensitized serum, his spermatozoa were examined under the microscope. While they were less plentiful than under normal conditions, there was considerable activity among them. However, many immobile ones were visible. Female 80, to which he was mated on this date, bore seven young. His spermatozoa were again examined on March 19th. This time they were still fewer in numbers and most of them were immobile. However, 52A1, mated to him on this date, bore eight young April 21st. Four young were born by 55A4, bred to him on March 20th. His seminal fluid examined on March 23rd was normal in quantity and still had the same milky look as normal semen, but comparatively few spermatozoa were to be seen in the field under the microscope, and practically all of these were immobile. A search for fifteen minutes revealed only two spermatozoa in motion, and these showed only feeble undulations of the tail. Bred to 30A3 on this date, he showed

normal mating vigor, but no young resulted from the union. On March 27th, his semen was again examined microscopically. Spermatozoa were more plentiful than at the last examination and some of them showed considerable activity. Neither numbers nor activity were back to normal, however. His mating to 84 on this date yielded six young. On May 13th he was bred to 6A2. His semen, though normal in quantity, was almost devoid of spermatozoa. The few which were present were wholly immobile. No young resulted from this mating. On June 1st he was mated to 31A3 without issue. His semen was almost free from spermatozoa. Here and there an immobile one was visible and very rarely a mobile one. June 12th, conditions were practically the same. A mating to 7A1 yielded no young.

This male (4A7) was then left with a female from June 12th until the middle of October, when he was killed for a study of his testes. Although he copulated with the female from time to time, no young were born. His semen was examined again July 3rd and seemed to be wholly devoid of spermatozoa. Upon removal, the testes were found to be somewhat smaller than usual for a rabbit of his size and somewhat flabby. Upon sectioning, however, no differences could be discerned between his seminiferous tubules and those of a normal male rabbit. The usual series of spermatogonia, spermatocytes, spermatids, and spermatozoa in all stages of transformation were in evidence. Moreover, some of the spermatozoa teased out of the epididymis and examined in normal saline solution were feebly motile, though most of them were inactive. In the case of this particular individual, at least, the sterilizing effects of the spermatotoxic serum apparently did not extend back beyond the mature spermatozoa.

From time to time tests were also made of the spermatotoxic action in vitro of the sensitized fowl-serum. For example, on March 3rd and March 4th the serum of fowl no. 1 was tested. Hanging-drop preparations were made of: 1) normal rabbit spermatozoa in normal saline solution; 2) normal rabbit spermatozoa in normal fowl serum, and, 3) normal rabbit spermatozoa

in spermatotoxic fowl serum. The spermatozoa in the spermatotoxic serum were largely immobilized at the end of ten minutes, and wholly so at the end of thirty minutes. The spermatozoa in normal fowl serum were still active at the end of an hour, when a gradual slowing down of motility set in. The spermatozoa in normal saline solution were moving about with undiminished activity at the end of three hours, when observations were discontinued.

To each of two test-tubes, one of which contained 2 cc. of spermatotoxic serum and the other 2 cc. of normal fowl-serum, a cubic centimeter of normal rabbit spermatozoa in normal salt solution was added. The tubes were then placed in an incubator at 38°C. and examined twenty-four hours later. The spermatozoa in the normal serum, though immobile, were intact; those in the sensitized serum were much fragmented, particularly tails and heads were separated as if a very soluble or digestible point existed in the region of the middle-piece. The same phenomena were observed in hanging-drop preparations similarly left over night. Also, there had been a very marked agglutination of the spermatozoa in the spermatotoxic serum. There had been some agglutination, though not so much, in the normal serum.

On March 4th the foregoing tests were repeated with the serum of the second sensitized fowl, with practically the same results.

On March 11th similar tests were made with the serum of the third sensitized fowl. This serum proved to be more active than that of the other two fowls. In a mixture of 1 part of the sensitized serum and 1 part normal salt solution the spermatozoa of a normal rabbit were practically all immobilized by the end of five minutes, although some were still alive at the end of an hour in a similar dilution of normal fowl serum. Various other dilutions were tried, as 1 part serum to 2, 3, and 4 parts of normal saline solution, respectively. In the 1-to-3 dilution of spermatotoxic serum the spermatozoa were immobile at the end of ten minutes; in the 1-to-4 dilution slight motion was

visible at one or two places of the microscopic field for over twenty minutes. In corresponding dilutions of the normal fowl serum motion continued for hours.

Experiment 2

In the meantime, in April, a new set of experiments was begun. April 1st five fowls were injected subcutaneously (two injections in one of the fowls were intravenous) with the semen from four male rabbits, obtained from females as in the earlier experiment. Sufficient normal saline solution was added to the semen to make a dose of 3 cc. for each fowl. One fowl died two days later. April 8th, the remaining four fowls were injected subcutaneously with the semen from three male rabbits, and this treatment was repeated on April 15th, April 24th, and May 1st. Two males, 25 and 83, were selected for tests with the spermatoxic serum. Both were first mated to test their fertility by breeding and by microscopic examination.

Male 25 was mated to 52A5 on April 13th (three young were born of this union) and microscopical examination of his spermatozoa showed them to be very active and plentiful. Samples placed in the sensitized fowl-serum were markedly agglutinated and all were immobilized within three minutes. He was given intravenous injections of the sensitized fowl-serum as follows: May 15th, 3.5 cc.; May 17th, 4 cc.; May 20th, 4.5 cc.; May 26th, 4.5 cc.; May 28th, 4.5 cc. On May 22nd his semen showed a reduced number of spermatozoa with many immobile ones in the field; however, others showed considerable activity. A mating to female 32A2 on this date yielded seven young. On June 12th, though much reduced in numbers, many of his spermatozoa as seen under the microscope were active. A mating to female 4A3 yielded no young. July 10th, while his spermatozoa were much reduced in numbers, some were still active, although most of those visible were immobile. Female 54A3, to which he was bred on this date, had no young. On October 6th his sperm was again examined microscopically. While reduced in quantity to what a rough estimate placed at one-fourth

normal when compared with the normal control, a much greater proportion of them were active than at the last examination. A mating to female 52A5 on October 6th yielded four young.

The spermatozoa of the other male, 83, were found to be normally plentiful and active when he was mated to 8A8 on May 13th. He was given intravenous injections of the sensitized fowl serum as follows: May 15th, 3.5 cc.; May 17th, 4 cc.; May 20th, 4.5 cc.; May 26th, 4.5 cc.; May 28th, 4.5 cc. On May 22nd, microscopic examination showed his spermatozoa to be about normal in number, though many immobile ones were to be seen. A mating to 32A3 on this date yielded seven young. On June 12th he was mated to 23B3 and six young were born July 14, one of which had both hind legs paralyzed. The semen of male 83 on June 12th was normal in quantity, though the number of spermatozoa was noticeably reduced. Many of the latter, however, were active. Conditions were about the same on July 3rd. A mating to 55A4 on this date resulted in the birth of eight young. A mating to 43 on October 8th yielded no young, although an examination of the semen still revealed some active spermatozoa.

A control male (untagged) was injected with normal (i.e., not sensitized) fowl-serum as follows: May 15th, 4 cc.; May 17th, 4 cc.; May 20th, 5 cc.; May 22nd, 4 cc.; May 28th, 5 cc. His semen, examined microscopically May 22nd, June 12th, and July 10th showed no diminution in numbers of spermatozoa nor in their motility. Matings on these dates resulted in litters of five, five, and seven, respectively.

Tests were also made of the cytotoxic activity in vitro of the respective sera of the four fowls used in this second experiment. The serum of the first fowl (May 13th) immobilized the spermatozoa of male 25 and male 83 within three minutes. Those of 25 were markedly agglutinated, those of 83 showed little or no agglutination. This serum was from the fowl which had received two intravenous injections of rabbit sperm. The serum of the second fowl (May 17th) immobilized normal spermatozoa within five minutes. Controls in normal fowl serum were still moving vigorously thirty minutes later, when observations were

discontinued. The serum of the third fowl (May 26th) immobilized normal spermatozoa in from five to seven minutes. In a 2-to-1 dilution of the sensitized serum with normal saline solution normal spermatozoa were immobilized in thirty minutes, while in a similar dilution of normal serum they were still active at the end of forty minutes. The undiluted serum of the fourth sensitized fowl immobilized normal rabbit spermatozoa in ten minutes; normal fowl-serum did the same in thirteen minutes. Sensitized serum of the fourth fowl, diluted with an equal volume of normal saline solution, immobilized normal rabbit spermatozoa in ten minutes; similarly diluted normal fowl serum did so in twenty-three minutes. Diluted with 3 parts of normal saline solution, this same sensitized serum immobilized normal rabbit spermatozoa in ten minutes; normal fowl-serum similarly diluted did so in thirty minutes. One part of the sensitized serum and 5 parts of normal saline solution produced immobility in such spermatozoa in seventeen minutes; in a similar dilution of the normal serum, some activity was observable nearly two hours later, when observations were discontinued.

In the meantime tests were also being made on the spermatozoa of a guinea-pig with the serum from the fourth fowl sensitized against the spermatozoa of the rabbit. This serum diluted with 5 volumes of normal saline solution immobilized the spermatozoa of the guinea-pig in twenty-five minutes; in normal fowl serum similarly diluted such spermatozoa were still active three hours later, when observations were discontinued. In a mixture of 1 part of the sensitized serum to 10 parts of normal saline solution, the spermatozoa of the guinea-pig were immobilized in a little over thirty minutes, while in the control of normal fowl serum similarly diluted they were still active three hours later.

Nearly a year later, April 5, 1921, males 25 and 83 were again tested for fertility. Male 25 had many active spermatozoa in his semen, although the total number was below normal. A mating made with him on this date yielded six young. The semen of male 83, however, when examined microscopically, appeared to be free from spermatozoa and the female bred to him bore no young.

Male 83 was killed April 11, 1921, and the testes were removed for further study. The right testis was of about average normal size, but the left was very small—not more than one-fifth the mass of the other. Sections were prepared from various regions of each testis. Although the right appeared normal when inspected superficially, microscopic examination revealed that it was entirely devoid of spermatozoa, spermatids, and secondary spermatocytes. Some of the seminiferous tubules contained only a peripheral layer of spermatogonia and generally these were reduced in number. In such tubules the field lying inward toward the lumen was usually occupied by a more or less loose fibrous reticulum. Other tubules showed numerous primary spermatocytes, but these rarely progressed in spermatogenesis beyond the contraction phase of nuclear change. Many tubules contained degenerating primary spermatocytes with dense, nondescript nuclear masses which stained intensely black with iron-hematoxylin. In some instances the entire microscopic field was filled with an indiscriminate mixture of necrotic nuclear and cytoplasmic substance, staining deeply with iron-hematoxylin.

Why the critical point at which deterioration becomes manifest should lie in the primary spermatocyte and whether it is concerned in any way with synapsis seems unanswerable at present. It is an interesting fact that in my earlier studies[3] on pigeon hybrids and on guinea-chicken hybrids, abnormalities in nuclear activity likewise came into evidence at the synaptic period of spermatogenesis. In the case of such hybrids I attributed the irregularities to incompatibilities between chromosomes of widely divergent parentage which prevented normal pairing of chromosomes. Such an explanation, even if true for hybrids, is obviously inadequate in the present instance. All that can be said with any assurance is that primary spermatocytes seem to be particularly susceptible to injurious influences of different kinds.

The left testis of rabbit 83 was entirely devoid of seminiferous tubules. It seemed to have been reduced to a nodule of dense

[3] Bul. 22, Univ. of Cincinnati, 1903. Jour. Morph., vol. 23, no. 1, March, 1912.

connective tissue. Traces of epididymis could be identified, but all germ cells had disappeared.

It is obvious from experiments 1 and 2 that fowls injected repeatedly with the spermatozoa of rabbits will produce a spermatotoxic serum that is violently toxic in vitro and also toxic to some degree in vivo. Of the three male rabbits (4A7, 25 and 83) which survived the treatment, 4A7, judging from the breeding test, had become completely sterile. However, his semen was entirely free from living spermatozoa for only a few weeks. No degenerative changes could be detected in his testes when sectioned and examined microscopically. Male 25 did not become sterile, at least for a prolonged period, according to the breeding test, although as a result of the treatment his semen showed a marked decrease in the number of inactive spermatozoa among those which were present. Male 83, however, not only became sterile, but exhibited a marked atrophy of the left testicle and pronounced degenerative changes in the seminiferous tubules of the right testicle. Spermatotoxins generated against the spermatozoa of the rabbit were also toxic in vitro for the spermatozoa of the guinea-pig.

ISOSPERMATOTOXINS AND AUTOSPERMATOTOXINS IN RABBITS

Experiment 3

The purpose of the remaining experiments was to determine if an animal can be made to produce antibodies against its own tissues. Spermatozoa of rabbits were used as the elements to be so tested.

Two male (32A4 and an untagged one) and two female rabbits (32A2 and 6A3) were employed in experiment 3. For each treatment the semen of several males was drawn off from females in the manner already described and mixed with sufficient normal saline solution to provide a dose of about 3 cc. for each rabbit injected. Injections were made into the marginal vein of the ear. The dosage was as follows: October 18th, semen from three males; October 25th, the same. Male 32A4 died immediately after the second injection, but whether from thrombus

formation or anaphylactic shock could not be determined. November 1st, the three remaining individuals were injected with the semen from three males, and this was repeated November 8th and November 15th.

The serum of the sensitized male (untagged) was tested November 22nd. The spermatozoa of this male and those of a normal male were first tested in normal rabbit serum diluted with 3 parts of normal saline solution. In this mixture the spermatozoa of the sensitized male had ceased activity at the end of three hours, while those from the normal male were still very active the next morning, fifteen hours later. It would seem from this that the intravenous injections of spermatozoa which the treated male had received had rendered his spermatozoa much less viable than those of an untreated rabbit.

Normal rabbit spermatozoa placed in the serum of the sperm-sensitized male were somewhat reduced in activity at the end of four hours and were wholly inactive next morning, fifteen hours later; normal spermatozoa in normal rabbit serum, used as a control, were still active at the end of twenty-four hours, when observations were discontinued. The spermatozoa of the treated rabbit when placed in his own serum were wholly immobilized by the end of seven minutes.

Tests made on normal spermatozoa with the blood of the sensitized female, 6A3, gave results similar to those obtained with the serum of the sensitized male. However, the spermatozoa of the treated male survived two hours and fifteen minutes in the serum of 6A3 as against seventy minutes in his own.

A duplicate set of experiments on the serum of this male and female yielded results so nearly identical to the first set that they need not be given in detail. Again the spermatozoa of the treated male survived longer (one hour) in the sensitized serum of the female than they did in his own serum.

On November 24th similar tests were made with the serum of the same male and with that of the sensitized female, 32A2. This female had borne four young November 18th, and the blood of one of these was also tested. Normal spermatozoa in normal rabbit serum were still in motion at the end of forty-six hours,

when observations were discontinued; in the serum of 32A2, however, they were immobilized in a little over an hour; in the serum of one of her young they remained active about thirty hours; in the serum of the treated male they had ceased activity by the end of forty-three minutes. The spermatozoa of the sperm-treated male were also tried out in the various sera. In his own serum they were immobilized in less than half an hour; in the sensitized serum of female 32A2 most of them were immobilized by the end of an hour, though a few were still moving feebly two hours later, when observations were discontinued for the day. Those placed in normal serum and in the serum of the young of 32A2 still showed considerable motion at this time, although their activity was noticeably more reduced in the serum of the young one. Both lots had ceased to move by the next morning, therefore the exact time at which each had become inactive was not determined.

The results of experiment 3 show that active spermatotoxins can be built up in rabbits against the spermatozoa of rabbits. Also, apparently, male rabbits injected with rabbit spermatozoa have their own spermatozoa greatly weakened. In this experiment the serum of the male became more toxic than that of the females, but it does not follow, of course, from a single experiment, that this is the rule. The serum of a young rabbit three days old, born of a sensitized mother, was toxic, but much less so than that of its mother.

Experiment 4

The experiment was next made to determine whether or not a given individual will form antibodies against its own spermatozoa when these are introduced into the blood. On December 8th males 58 and 29A3 were bred to separate females and each was then injected intravenously with his own semen. On December 15th this was repeated with 58, but 29A3 seemed unable to mate and he was abandoned. Male 53B2 was mated instead (December 16th) and injected. On December 18th, 58 was mated again and injected as before with his own semen.

December 26th blood was drawn from 58 and from a normal male and the sera tested. Normal spermatozoa in the serum of 58 were completely immobilized at the end of twenty-four hours; in the normal serum they were still moving considerably at the end of thirty-two hours, when observations were discontinued. The spermatozoa of 58 when placed in his own serum showed no movement after eleven hours, but in normal serum they showed activity, though very feebly toward the last, for over twenty-four hours. From this test it was evident that rabbit 58 was forming spermatotoxin against his own spermatozoa.

On January 7, 1921, 58 and 53B2 were each given an injection of his own spermatozoa. About two hours later 53B2 became paralyzed in the hind legs, and as he did not recover he was killed January 17th. Before he was killed his blood serum was tested. Normal spermatozoa in this serum were for the most part inactivated by the end of six hours. In the control of normal spermatozoa in normal serum considerable activity was still in evidence at the end of thirty hours, when observations were discontinued.

On January 14th rabbit 58 was again injected with his own spermatozoa, and this treatment was repeated on January 21st and 24th, respectively. Thus male 58 received eight intravenous injections of his own spermatozoa in all. His blood-serum and his spermatozoa were both tested February 3rd. Normal spermatozoa in the serum of 58 were mostly immobilized at the end of six hours, although a few maintained feeble movement for twenty-four hours. In the control of normal spermatozoa in normal blood-serum, many of the spermatozoa were fairly active at the end of thirty-four hours when observations were discontinued. The spermatozoa of 58 in his own serum were wholly immobilized at the end of five hours, while immobilization did not occur in normal rabbit blood-serum until the end of twenty-five hours.

Male 32A1, substituted for the male (53B2) which developed paralysis of the hind legs, was injected with his own spermatozoa on the following dates: January 17th, 24th, 31st, and February 7th. His spermatozoa and blood-serum were tested February

17th. A microscopical examination of the fresh semen showed that the spermatozoa were reduced in number and that many of them were immobile. Normal spermatozoa were immobilized in the serum of 32A1 in three and a half hours, although they were still active in normal serum at the end of twenty-four hours. The spermatozoa of 32A1 in his own serum were immobile at the end of two and one-half hours; in normal serum, at the end of four and one-half hours.

From the foregoing experiments it is evident that not only does the blood serum become toxic for spermatozoa as the result of intravenous injections of the animal's own spermatozoa, but the living spermatozoa of such treated males also are affected in some way, since they are much less viable, even in normal serum, than are the spermatozoa of the untreated males. It was reasoned that this might be due to a specific effect in vivo of the spermatotoxic serum on the spermatozoa in question, or it might be simply the result of a general lowering of the animal's vitality, due to the introduction of considerable quantities of a foreign or an unaccustomed protein into the blood stream. To test this point spermatozoa from a male (90A2) which had received several intravenous injections of typhoid vaccine, followed by two heavy intravenous injections of living typhoid germs, were employed. The blood of this male was at its highest titre for the Widal reaction when used in the present experiment, March 3rd. For the test equivalent quantities of the spermatozoa of the male (90A2) treated with typhoid germs, the male (32A1) with spermatotoxic serum, and a normal male (4A8) were taken. In the spermatotoxic blood-serum of 32A1, his own spermatozoa were all immobilized at the end of twenty hours, but the spermatozoa of the other two males still showed some activity at the end of thirty hours, when observations were discontinued. In the serum of the normal rabbit the spermatozoa of 32A1 showed but little activity at the end of twenty hours, while those of the other two males were still active and remained so for hours. In the serum of 90A2, the rabbit treated with typhoid germs, the respective results with the three sets of spermatozoa were the same as in normal serum. Obviously, the

results of these tests indicate that the vitality of the spermatozoa of the sperm-treated males has been impaired in vivo by the spermatotoxic serum rather than as the result of a general constitutional weakening due to the presence of a foreign protein in the blood.

From time to time, in testing the spermatotoxic sera of various males, it was observed that while the great majority of spermatozoa were speedily immobilized in such sera, a few would flicker feebly for hours longer. It was inferred that this was due to the using up of the alexin (complement) of the spermatotoxic serum in the immobilizing reaction. With this gone, there was no reason why the spermatozoa should not live as long in such serum as in normal serum. To put the matter to a test, spermatotoxic serum was drawn from male 32A1, February 17th, and divided into two parts. One of these was 'inactivated' (alexin destroyed) by heating it to 56°C. for thirty minutes. A control of normal serum was also used. Normal spermatozoa subjected to the spermatotoxic serum of 32A1 were immobilized by the end of three and one-half hours, but were still moving in the inactivated spermatotoxic serum and in the normal serum twenty-four hours later when observations were discontinued. The spermatozoa of 32A1 were immobilized in his own serum at the end of two and one-half hours, although in his inactivated serum they continued to be very active at the end of this time and still showed considerable motion at the end of seven hours.

Various investigators have described degeneration of the seminal epithelium in different mammals due to occlusion or resection of the ductus deferens. Kuntz,[4] for instance, finds that ligature and resection of the right ductus deferens in dogs induces degeneration of the seminal epithelium not only of the right but in the left testis as well. In view of the fact, established through the present investigation, that an animal will develop a toxin in its blood serum poisonous to its spermatozoa and germinal epithelium when its own spermatozoa are introduced into its blood stream, the possibility suggests itself that the degenerative changes in seminal epithelium which follow

[4] Anat. Rec., vol. 17, no. 4, Dec. 20, 1919.

resection or occlusion of the ductus deferens may similarly be due to the action of a spermatotoxin. For spermatozoa which could not leave the testis must ultimately die and be resorbed, and it seems probable that upon their resorption spermatotoxins would be formed which could attack the living germ-cells of the testis. Depending upon the virulence of such toxin, complete or partial sterility might ensue. However, that occlusion of the efferent duct of one testis does not always prevent the formation of spermatozoa by the other testis—at least permanently—is evident from the many cases known in man where an orchitis or an epididymitis of one testicle has resulted in such occlusion on one side, and yet the individual has remained fertile.

SUMMARY

1. Spermatotoxic sera, prepared by injecting fowls repeatedly with the spermatozoa of rabbits, are toxic in vitro for the spermatozoa of both rabbits and guinea-pigs.

2. When introduced into the blood stream of male rabbits at intervals for four or five weeks, such serum produced partial or complete sterility. Inactivation of many spermatozoa, reduction in numbers, or even complete disappearance from the semen occurred. In one case the sterility was partial or temporary; in a second, complete, when judged by the breeding test, although the germinal epithelium appeared to remain normal and a few spermatozoa were visible; in a third, complete, accompanied by marked degenerative changes in the testicles.

3. Microscopical examination of the testes of the latter individual (rabbit 83) showed that not only were the mature spermatozoa affected, but disintegrative changes had taken place or were in progress in the seminiferous tubules.

4. The blood-serum of a rabbit injected intravenously with its own spermatozoa becomes highly toxic for the spermatozoa of rabbits, including its own.

5. The spermatozoa of a rabbit which has been repeatedly injected with its own semen are much less viable, both in normal rabbit serum and in spermatotoxic serum, than are normal spermatozoa. Presumably such spermatozoa have been in-

fluenced specifically in vivo by the spermatotoxic serum of their own host.

6. Since an animal can thus on occasion build antibodies against its own tissues when these have become misplaced or altered, and since antibodies can directly or indirectly affect the germ-cells, it is reasonable to suppose that such influences may be the source of certain germinal variations.

Resumen por la autora, Bessie Noyes.

Estudios experimentales sobre el ciclo vital de un rotifero que se reproduce por partenogénesis (Proales decipiens).

El presente trabajo es una descripción del ciclo vital normal del rotífero Proales decipiens, incluyéndose en él resultados que demuestran la ausencia de una disminución del vigor de la raza estudiada durante 250 generaciones partengenéticas. En el habit natural de la especie no se han encontrado machos, ni tampoco aparecieron cuando se modificaron experimentalmente las condiciones ambientes con relación a la naturaleza y concentración de los alimentos, su constitución química y la temperatura. Con el intento de aumentar la puesta de los huevos la autora ha llevado a cabo la selección durante tres meses y también con el intento de aumentar la duración media de la vida obteniéndose en ambos casos resultados negativos, mientras que el tratamiento por el alcohol etílico en 1/4 por ciento y 1/2 por ciento de concentración durante veinte semanas influyó muy poco sobre la duración de la vida, aun cuando hubo una reducción del número de huevos producido. Esta reducción no fué retenida por la progenie más allá de la tercera generación al volver a las conditiones normales de cultivo.

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EXPERIMENTAL STUDIES ON THE LIFE-HISTORY OF A ROTIFER REPRODUCING PARTHENOGENETICALLY (PROALES DECIPIENS)

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CONTENTS

INTRODUCTION

Proales decipiens, a small rotifer belonging to the family of Notommatidae, presents unusual opportunities for studies on the life-history of the Rotifera, for work on long-continued parthenogenesis, and particularly for attempts to alter racial characteristics by selection or by environmental action. The animal lives in cultures of decaying vegetable material; it thrives in the hay infusions, malted-milk solutions, and the like, commonly employed for cultures of Paramecium. It is extremely hardy, readily lends itself to culture of isolated individuals on hollow-ground slides, and thrives under wide ranges of environment. At laboratory temperature it begins to produce eggs twenty-four to thirty-six hours after hatching. The number is low on the first day, increases gradually until the fifth day, then declines sharply, and egg production ceases on the seventh or eighth day, and the death of the adult follows in one or two days. In a typical life-cycle an egg deposited by the mother hatches in twelve to twenty-four hours; the embryo reaches the egg-laying period in twenty-four to thirty-six hours after hatching and

deposits on the average one egg the first day, three the second, five the third, seven the fourth, three the fifth, one the sixth, and death occurs on the seventh day of maturity.

The organism is cylindrical, soft, slender, and worm-like, with an undeveloped foot and small toes; the eye is small, red, and located on one side of the brain. The color varies from white in young specimens to dark brown in mature forms near the close of the life-cycle. Movement through the water is rapid for the first two or three days of the life-period, but gradually the size of the organism increases, a substance of a dark brown color is deposited within the body, and movement becomes increasingly sluggish. As ordinarily found, the animals are all females and reproduction is entirely by parthenogenesis.

Thus the creature furnishes us a hardy animal, readily cultivated in small compass, passing through the cycle of birth, infancy, maturity, old age, and natural death within eight days, and yielding within that time a large number of progeny, produced without that admixture of germ cells from diverse parents which so greatly complicates the problems of heredity and variation in most organisms. It has in these respects the advantages of an infusorian reproducing vegetatively, with the important diversity that here we have opportunity to study uniparental inheritance through germ cells.

The present paper gives an account of the normal life-cycle, with appropriate vital statistics; an experimental and observational study of the question as to the appearance of the male sex and fertilization; a study of variation and the results of selection during parthenogenesis, and an account of attempts to alter the hereditary characteristics through the action of environmental agents.

All the organisms studied have descended from one individual isolated by Dr. H. S. Jennings during the first week in February, 1919, with the exception of five 'wild' specimens which were later found and cultivated for the sake of comparison.

Proales will live and reproduce in cultures containing decaying vegetable material, in hay infusion, in solutions of pasteurized milk, and other similar media, but in the present work it was

found that the greatest degree of uniformity could be obtained with a solution of malted milk. A stock solution of $\frac{1}{16}$ of 1 per cent strength was made from Horlick's Malted Milk in powder form as follows: $\frac{1}{2}$ gram of the powdered milk was weighed and put in a glass beaker; approximately 5 cc. of boiling water was added, and the powder stirred until in suspension and free from lumps, when the remainder of the 100 cc. of boiling spring water was added. This solution was than put into a liter graduate and filled to 800 cc. with filtered cold spring water. A still greater degree of uniformity in culture medium could have been obtained with distilled water, but Proales did not thrive well in cultures made in this way.

In a study of individual performance, or in pedigreed cultures, a single organism was isolated in 5 drops of culture fluid in each of the depressions of a hollow-ground slide; these slides were kept on glass plates in large covered Stender dishes partly filled with water to prevent evaporation of culture fluid. Reserve mass cultures were always kept in small watch-glasses with about 5 cc. of malted milk, changed once each week.

The early deposition of eggs, together with the rapidity with which these eggs hatched and produced other egg-laying individuals in their turn, necessitated the examination of all slides once in every twenty-four hour period, and if individual data were to be obtained for the eggs it was necessary to remove each to a separate depression. Various modifications of the general method were made to meet the requirements of the different changes in environment, but these can best be discussed in the section to which they particularly belong.

THE NORMAL LIFE-HISTORY

The normal life-cycle of Proales is complete within eight days, although great variation occurs even when the organisms are kept under constant conditions of temperature and food. Early in the history of the race (second week of February, 1919) among 100 isolated individuals cultivated in $\frac{1}{16}$ per cent malted milk at laboratory temperature, 69 lived for 5 days, 27 for 4 days, and 4 for 3 days, giving an average length of life of 4.65 days.

Five months later (in June, 1919), among another 100 organisms isolated under the same experimental conditions, 42 lived for 7 days, 39 for 6 days, 15 for 5 days, 2 for 4 days, and 2 for 3 days, giving an average length of life of 6.17 days. This increase in the length of life, accompanied by a marked increase in the number of eggs deposited, formed the basis for an experiment in selection in an attempt to increase both of these factors beyond the limits indicated above, as detailed in a later section. A tabulation of 1200 individuals reared at laboratory temperature (47° to 63°F.) in $\frac{1}{16}$ per cent malted milk at various times during the study shows that 295 lived for 7 days, 357 for 6 days, 382 for 5 days, 127 for 4 days, 35 for 3 days, and 4 for 2 days, giving an average length of life of 5.60 days (table 1). Under these conditions, with food constant and temperature variable, the individual range of life was from two to seven days with an average life cycle of 5.47 days for 1400 individuals. In an experiment with 254 organisms isolated in $\frac{1}{16}$ per cent malted milk and kept constantly at 23° to 25°C., it was found that 9 lived for 7 days, 56 for 4 days, 84 for 5 days, 60 for 4 days, 33 for 3 days, 10 for 2 days, and 2 for 1 day, giving an average length of life of 4.68 days.

TABLE 1

A table of the length of life of 1200 individuals (100 in a group) reared in $\frac{1}{16}$ per cent malted milk at laboratory temperature

2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS	7 DAYS	TOTAL
	4	27	69			100
	2	2	15	39	42	100
	8	26	66			100
	2	2	15	39	42	100
2	5	19	34	29	11	100
	2	4	27	38	29	100
	2	8	20	29	41	100
	3	9	23	34	31	100
	2	5	29	33	31	100
1	2	10	24	34	29	100
1	3	7	32	42	15	100
		8	28	40	24	100
Total, 4	35	127	382	357	295	1200

Thus, under conditions of constant temperature and food, the average length of life is lower than the average for individuals kept under conditions of constant food supply and fluctuating temperature.

As all these records were from isolated individuals, in many of the cases the short life of an individual may have been due to a slight mechanical injury inflicted when the organism was transferred from one depression slide to another in the daily routine; this is particularly likely to occur towards the close of the life-cycle when the posterior part of the body has become inflated and lost the power of movement.

When the egg is deposited in the water there is no indication of movement in the embryo; but six to eight hours before hatching the embryo is clearly distinguishable and can be observed as it turns within the egg; the jaws are in constant motion, striking out against the membrane apparently in an attempt to force a place of exit. Just before the individual emerges the egg membrane bulges outward near the larger end of the egg and immediately over the jaws of the embryo; this bulge increases until the membrane ruptures, leaving an irregular opening through which the organism escapes. The first definite bulging of the egg membrane occurs about two minutes before the organism escapes into the water. After emerging, the young individual moves rapidly, at random, through the water, twisting and bending sharply in its course. The body increases gradually in length and diameter for the first four days. At this time the maximum length is reached in most cases, but the increase in diameter continues until a day or two before death.

At room temperature the organism begins to deposit eggs about twenty-four hours after it has hatched, and one to two days before the maximum length of the body is attained. At this time a yellow-brown substance begins to appear in the posterior three-quarters of the body; this substance increases in amount until the body becomes brownish in color, the posterior part becomes inflated, and most of the power of movement is lost, owing to the increase in size and the loss of flexibility. In isolated individuals random movement ceases about the second

day of the egg-laying period and the adult takes a circular path near the edge of the medium; as a result of this course, the eggs are deposited near the edge of the liquid and usually occur in two or three groups. The act of depositing eggs has never been observed, so that it is not known whether these groups represent two or three separate deposits or whether the eggs are deposited singly and the groupings are accidental and the result of the circular path taken up by the mother. In the young, transparent organism the passage of food through the jaws, through the esophagus and back into the body, and finally the excretion of waste products can be traced with ease. About the time of death, however, excretion ceases, movement is confined to the anterior cilia and jaws, and the action of the jaws in grasping food seems to be ineffective. Throughout the entire life-cycle the extrusion of waste products from the body seems much less than the amount of material taken through the jaws would indicate, and during the last two days of the life-period many specimens cease the extrusion of fecal masses entirely. At the close of the life-cycle the organism differs markedly from the adult early in the egg-laying period.

The organism furnishes excellent material for a study of the physiological and structural changes accompanying maturity, old age, and death. The transparency of the embryo with its rapid development and the accompanying change in color offers excellent opportunity for a study of the relations of the retention of the products of metabolism, muscular degeneration, and the effects of aging.

The usual type of egg deposited by Proales is slightly larger at one end, whitish in color, very thin-shelled, and $48 \times 80\mu$ in size. The egg increases very little in size before hatching, but a few hours after it has been deposited the outline of the embryo can be distinguished. This outline increases in distinctness, and soon the embryo can be seen flexed on itself with the long axis parallel to the long axis of the egg. In over 50,000 eggs observed in fresh culture medium, hatching has taken place in all cases within twenty-four hours, with the exception of five individual eggs which appeared to have been slightly injured

when the mother was removed from the slide and which disintegrated very soon. If eggs are left in isolation slides in old culture medium, hatching frequently does not take place at all; a cloudy, irregular, zone appears around the egg and disintegration soon follows.

In February, 1919, immediately after the study was begun, sixteen eggs quite different from the usual type described above were deposited. These eggs were $90 \times 150\mu$ in size, yellowish brown in color, with a lighter yellow circular area in the center, blending to a dark brown at either end. The shell or egg membrane was very thick. All of these eggs were kept in fresh culture medium for three months, under conditions in which the thin-shelled forms hatched readily. No change in appearance had taken place during this time, so they were separated into two lots; one lot was subjected to a temperature of $\pm$ 6°C. for a period of two days, then returned to the temperature of the laboratory; the other lot was kept at 36°C. for an equal length of time and returned to the laboratory temperature, but no change followed this period of increase and decrease in temperature. Finally, all the eggs were dried for two weeks at laboratory temperature and then returned to fresh culture solut on, but as no change could be detected, they were discarded at the end of six months. No eggs of this type have appeared during the later study of the organism, lasting over a period of thirteen months.

In many species of rotifers two kinds of eggs are produced, thin-shelled ones during periods favorable for rapid multiplication, and thick-shelled forms at the onset of unfavorable conditions; these later are known as the 'winter' or 'resting' form. In Hydatina senta, studied so extensively by Whitney and by Shull, three forms are produced, a large thin-shelled form which develops at once into a female, a small thin-shelled which develops at once into a male, and a thick-shelled form which develops after a period of rest into a female; the first two forms are produced parthenogenetically, the third by a fertilized female. The thick-shelled eggs of Proales are comparable to the 'winter' eggs described above, since they appeared at a time when reproduction under normal conditions might be accomplished

with difficulty; but such a comparison must be made with reservation, owing to the absence of any such eggs during the winter of 1919–1920, although some cultures were under the same conditions of environment as during the previous winter. On the other hand, such thick-shelled forms may have been deposited by fertilized females and have never appeared in later cultures, since all forms produced in malted milk have been females and there has hence been no possibility of fertilization taking place.

An individual usually deposits an egg during the first twenty-four hours of its life, and the average daily rate of production determined for 1500 individuals isolated in $\frac{1}{16}$ per cent malted milk was 1 egg the first day, 3 the second, 5 the third, 7 the fourth, 3 the fifth, and 1 the sixth. There is, however, much variation in different individuals, as will be seen from table 2, which gives the daily egg production for fifty specimens in February, 1919, and for fifty specimens five months later in June, 1919.

As the table shows, there is an individual range of about 1 to 10 eggs for every day of the egg-laying period. If the number of eggs deposited on the first day is high (7 to 9), the life-cycle is usually shortened to three to five days, but if the number is small (1 to 3) on the first day and increases gradually on the second and third days, the life-cycle is longer (five to seven days) and in many cases the egg production higher. For 1200 individuals in $\frac{1}{16}$ per cent malted milk at room temperature (47° to 63°F.) the average total egg production was 19.56. For 254 individuals in $\frac{1}{16}$ per cent malted milk at a constant temperature of 23° to 25°C. the average total egg production was 17.37. This decrease in number of eggs was associated with a decrease in the average length of life from 5.60 days to 4.68 days, as has been noted earlier in the paper. For 100 individuals isolated early in the study the average total egg production was 12.79, while the average production of an equal number of individuals a few months later was 23.43 eggs per individual. This increase in average egg production was associated with an increase in the average length of life from 4.65 days to 6.17 days. This marked difference in egg production and length of life in two groups of

organisms at different times in the history of the race suggested the possibility of increasing these two factors still further by artificial selection; an experiment with this in view is described later.

The early production of eggs, the large number of eggs produced, the high percentage of viability in fresh malted-milk solution, together with the short length of the average life-cycle, give us a race of organisms increasing rapidly. In 100 trials made during the study, a single individual, isolated in a small watch-glass, supplied daily with fresh malted milk and allowed to reproduce for seven days, showed an average of 125.63 descendants (adults, young, and eggs), including members of four generations. During the thirteen months the race has been under observation over 125,000 individuals have been studied in isolation; all of these organisms have produced eggs of the thin-shelled variety, with the exception of the sixteen thick-shelled forms mentioned earlier. The fact that only females had been produced and the absence of the two types of eggs in the later work led to a search for the male form and the experimental conditions under which it appears, as set forth in the next section.

DO MALES OCCUR? AN EXPERIMENTAL STUDY

As has already been set forth in this paper, the line of descent in Proales consists mainly of females reproducing parthenogenetically. Is this the only method of reproduction? Do males ever appear, as in most other species of rotifers? In a number of species the male occurs either continuously or at irregular intervals in the life-history, but in one large group, the Bdelloida, no males are known and reproduction takes place continuously by parthenogenesis. In the genus Proales males have been observed for two of the four species; in Proales werneckii, which lives in galls of Vaucheria, the male resembles the female closely in size and form, although the alimentary canal is not so fully developed; in Proales parasita, Plate has recorded the appearance of a male of the usual reduced size and structure; in Proales decipiens and Proales gigantea the male has not been recorded.

TABLE 2

Daily egg production of 100 individuals of Proales decipiens, cultivated in $\frac{1}{16}$ per cent malted milk

A. FIFTY INDIVIDUALS IN FEBRUARY, 1919							B. FIFTY INDIVIDUALS IN JUNE, 1919								
INDIVIDUAL	Days						INDIVIDUAL	Days							
	1st	2d	3d	4th	5th	Total		1st	2d	3d	4th	5th	6th	7th	Total
1	5	3	3	d		11	1	2	2	6	7	5	5	d	27
2	4	2	2	2	d	10	2	1	5	7	5	4	2d		26
3	3	1	1	2	4d	11	3	1	4	8	6	3	1	d	23
4	1	1	2	3	d	7	4	1	7	6	6	4	2	d	26
5	4	2	3	1	1d	11	5	2	6	5	7	3	1	1d	25
6	5	1	2d			8	6	1	8	7	5	4	2d		27
7	7	3	d			10	7	3	6	7	d				16
8	8	4	0	2	1d	15	8	2	7	6	4	1	d		20
9	9	6	0	4	2d	21	9	2	6	7	6	3	2d		26
10	0	0	10	2	d	12	10	4	6	7	8	2	d		27
11	2	2	3	3	11d	21	11	3	6	8	7	2	1	d	27
12	12	2	2	2	1d	19	12	2	6	6	7	4	2	1d	28
13	1	3	3	4	1d	12	13	1	5	7	5	2	1d		21
14	2	1	2	2	d	7	14	2	9	5	7	3	0	d	26
15	2	2	6	1	2d	13	15	2	8	4	3	3	3d		23
16	1	1	3	3	2d	15	16	1	9	5	2	1d			18
17	5	3	0	d		8	17	1	5	6	7	4	3	d	26
18	2	3	0	3	1d	9	18	2	6	6	3	2	1d		20
19	4	1	5	4	4d	20	19	1	7	8	9	1	d		26
20	1	4	5	3	2d	15	20	0	8	8	7	4	2	1d	30
21	1	2	5	4	1d	13	21	0	7	5	3	d			15
22	5	4	3	4	2d	18	22	3	9	7	6	2	1d		28
23	0	3	3	4	1d	11	23	2	6	7	8	3	2	1d	29
24	3	4	2	4	1d	14	24	1	5	9	8	2	1d		26
25	2	3	6	5	2d	18	25	3	6	5	4	6	0	3d	27
26	0	2	4	6	3d	15	26	1	7	6	3	6	2d		25
27	3	4	5	6d		18	27	2	5	7	6	4	1	d	26
28	0	4	2	7	3d	16	28	5	6	4	3	1d			19
29	2	3	7	1	2d	15	29	1	6	4	5	7	0	d	23
30	1	3	5	4	d	13	30	4	7	5	4	5	0	d	25
31	1	3	2d			6	31	3	8	2	6	5	1	1d	26
32	1	2	6	8	3d	20	32	2	7	7	7	5	d		28
33	1	4	5	6d		16	33	2	6	5	4	1d			18
34	1	2	6	8	d	17	34	2	6	9	5	2	4d		28
35	0	3	4	5	6d	18	35	2	6	5	5	4	0	d	22
36	1	4	4	6d		15	36	1	7	8	6	5	0	d	27
37	1	4	4	8	1d	18	37	3	4	9	5	5	01d		27
38	3	2	5	6d		16	38	1	2	5	9	5	1	1d	24

TABLE 2—*Concluded*

A. FIFTY INDIVIDUALS IN FEBRUARY, 1919							B. FIFTY INDIVIDUALS IN JUNE, 1919								
INDIVIDUAL	Days						INDIVIDUAL	Days							
	1st	2d	3d	4th	5th	Total		1st	2d	3d	4th	5th	6th	7th	Total
39	1	3	6	6	2d	18	39	2	7	6	5	4	d		24
40	1	2	0	7	3d	13	40	1	8	7	5	3d			24
41	1	3	3	7	1d	15	41	1	9	10d					20
42	1	2	1	2	4d	10	42	1	3	5	7	4	2	1d	23
43	1	4	4	6	d	15	43	1	7	6	8	2	0	d	24
44	1	1	1	1	d	4	44	2	5	6	6	4	1d		24
45	1	3	0	d		4	45	3	6	9	7	1d			26
46	1	4	5	6	7d	23	46	1	10	10	2	1	0	d	24
47	1	4	6	8d		19	47	3	7	10	3	2	1d		26
48	1	3	4	5	2d	15	48	2	9	9	5d				25
49	1	3	5	7d		16	49	5	6	9	5	1	0	d	26
50	0	2	1	2d		5	50	3	9	9	6	2	0	d	29

d indicates day of death.

In most of the species of rotifers, the males differ markedly from the females; they are smaller, shorter-lived, much more active than the younger females, and are usually degenerate in structure, lacking complete excretory or digestive systems. But in Proales werneckii the male is of the same size as the female and resembles her externally. On this account it seemed inadvisable to rely on appearances alone as to whether the form under observation in Proales decipiens was male or female, but if on isolation eggs were deposited it was clear that the individual was female. The appearance of two types of eggs, thin-shelled and thick-shelled, early in the history of the race suggested that at that time males might be present and the thick-shelled eggs deposited by a fertilized female. This suggestion was supported further by the fact that the thick-shelled eggs did not appear during the second winter, as they perhaps would had they been merely 'winter eggs.' Early in the experiment it was noticed that in the usual culture fluid of $\frac{1}{16}$ per cent malted milk, all individuals isolated for any experiment produced eggs; that is, in malted milk no males appeared. In the case of

Hydatina senta, Shull and Whitney have shown that various factors in the environment have an influence upon the proportion of males appearing in any line. The amount of oxygen in the medium, the concentration of the food, the proportion of various microorganisms available for food, and the concentration of various chemicals seem to be factors associated with the percentage of males, or of male-producing females, produced by various mothers.

In Proales, changes in the nature and concentration of the food, changes in the temperature at which various cultures were maintained accompained by changes in food, and changes in the chemical constitution of the medium were introduced in the study of the conditions under which the male form might be produced. In a culture fluid of $\frac{1}{16}$ per cent malted milk, female forms had consistently appeared throughout the study; so increases in the concentration of the milk were made until solutions of $\frac{1}{8}$ per cent, $\frac{1}{4}$ per cent, $\frac{1}{2}$ per cent, and 1 per cent had been used. In the higher concentrations bacteria developed so rapidly that the medium soon became flocculent and less favorable for a high egg production and greater length of life. On the other hand, a decrease in the concentration of the milk to $\frac{1}{32}$ per cent and $\frac{1}{64}$ per cent tended to decrease the available food to such an extent that the number of eggs deposited decreased, although the length of the life-cycle was not influenced. Males do not appear in malted milk either in decreased or increased concentrations. Beef extract, made from Armour's bouillon cubes, was made in $\frac{1}{8}$ per cent, $\frac{1}{4}$ per cent, and $\frac{1}{2}$ per cent concentration. The number of eggs produced in any of these percentages was much lower than in the stock solution of malted milk and in no case was there any change in the nature of the eggs or the organisms produced. Pasteurized milk in $\frac{1}{16}$ per cent, and $\frac{1}{8}$ per cent concentrations was employed, but owing to rapid bacterial action the solution became flocculent within a very few hours at laboratory temperature; successive generations of rotifers were maintained in it with difficulty and no males were produced. A solution of horse manure made after Whitney's ('14) formula was prepared and used in the proportion of 1 part horse-manure

solution to 3, 4, 5 and 6 parts of spring water. Egg production and the average length of life were both much reduced in this medium and no change in the mature form or egg were produced. At various times throughout the experiment Proales was cultivated in spring water in which various unicellular green plants and animals were flourishing, but in all cases isolated adults produced eggs of the usual thin-shelled form. In all cases isolations were made for five successive generations, since Shull ('12) has shown that the action of the environment is not accompanied by a change in sex until grandchildren are produced. None of the changes in the nature or concentration of the food were accompanied by the appearance of the male form in Proales.

Cultures in $\frac{1}{16}$ per cent malted milk, $\frac{1}{4}$ per cent beef extract, and in $\frac{1}{16}$ per cent pasteurized milk were subjected to a constant temperature of $\pm$ 6°C., 17°C., 24°C., and to a fluctuating temperature of 13° to 23°C. without producing any change in the uniform female constitution of the population.

In the summer of 1919, a number of mass cultures were established in small watch-glasses; in each of these cultures there was 5 cc. of malted milk with sufficient of a $\frac{1}{10}$ N solution of the following chemicals to give the percentage (of $\frac{1}{10}$ N) indicated.

HCl, $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2, 3 per cent	KCl, $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1 2, 3 per cent
H_2SO_4, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2, 3 per cent	$MgSO_4$, 2, 4, 6 per cent
$NaCl$, 2, 4, 6 per cent	K_2HPO_4, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2, 3 per cent
$C_2H_4O_2$, $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2, 3 per cent	$NaNO_3$, 2, 4, 6 per cent

At various times isolations were made from each culture; but eggs were deposited by all of the individuals and there was no indication of the appearance of the male form.

Thus, neither a change in the kind nor the concentration of the culture medium, a constant or fluctuating change in the temperature, or a change in the chemical constitution of the medium, has been accompanied by the appearance of the male in Proales decipiens.

In June, 1919, five 'wild' specimens of this species were found in a culture jar filled recently with water from a small stream. These organisms were transferred gradually to malted milk, and

twenty-five eggs from the second generation in malted-milk solution isolated from each individual. All individuals thus separated deposited eggs of the usual thin-shelled type and there was no indication of the appearance of the male form.

To sum up, all individuals from the original clone have deposited eggs; no males have appeared during changes in chemical constitution of the medium, changes in temperature, or changes in kind and concentration of food. Progeny of individuals recently isolated from 'wild' ancestors likewise show a uniform production of females.

So, during the thirteen months that Proales decipiens has been under observation, reproduction has been entirely by parthenogenesis. Although no records have been kept of the generations produced by mass cultures during this entire period, an estimate of over 250 generations is conservative, calculated from records of sixteen to twenty-three generations produced from single ancestors during a period of thirty days' isolation. In this species continuous parthenogenetic reproduction for this number of generations has been accompanied by no evidence of weakening in the race. At the close of the experiment the range of egg production for100 individuals in $\frac{1}{16}$ per cent malted milk at laboratory temperature was from 11 to 30 with an average of 19.50; the range of the length of life was 4 to 7 days, with an average of 5.80 days. One hundred individuals early in the history of the race under the same conditions, at about the same time in the year, showed a range in egg production of 4 to 23, with an average of 12.79 and a range in the length of life of 3 to 5 days, with an average of 4.65 days. The hatching of eggs in fresh culture medium takes place within twenty-four hours in 100 per cent of the cases, and, barring accidents, the young individual begins to deposit eggs within twenty-four hours after it is hatched. While no definite measurements can be made of the body size of the adult, owing to its constant movements, the dimensions reached by the adult in the later generations are as great as those shown by any of the individuals produced in the early history of the race. Continuous parthenogenesis for about 250 generations in Proales, then, has been accompanied by no

reduction in the viability of the eggs, by no retardation in the development, by no reduction in the range or average of egg production, by no reduction in the range or average of the life-cycle, and by no reduction in the size attained by the adult.

THE EFFECT OF SELECTION

As we have already seen, there is great variation in the length of life and the number of eggs produced in the different individuals of Proales, the life-cycle ranging from one to eight days, the egg production from one to thirty.

Are these variations the results of constitutional and hereditary differences, so that, if individuals showing the different rates of egg production and differences in length of life are isolated and allowed to reproduce we shall get stocks differing consistently in these respects?

This question of variation and the inheritance of variation in uniparental reproduction has been much studied, especially since Johannsen ('03), working with self-fertilizing beans, reached the conclusion that selection within the progeny of a single individual is ineffective and that complete regression occurs in the progeny of all individuals showing variation from the mean for the pure line. The same condition has been found not only in self-fertilizing plants, but in plants reproducing vegetatively by tubers, by grafting, and by buds and in animals reproducing asexually by budding (Lashley, in Hydra), by fission (Jennings, in Paramecium), and by parthenogenesis (Ewing, in Aphids).

By analyzing Johannsen's results statistically, Pearson ('10) found indications that inheritance might exist within the clone, since the correlation ratios diminished as the line of ancestry became more remote. If the genetic constitution of any individual of a pure line does not depend on its immediate ancestor, but on the type of the line to which they both belong, the coefficient of correlation between any individuals and any generation of their ancestors should be the same as the correlation between the individuals and their immediate ancestors. Later experimental work on Protozoa has given data agreeing with the conclusion that selection within a pure line is to a certain extent

effective in some cases. In Stylonychia, Middleton ('15) isolated from the progeny of a single individual two strains that differed in the rate of fission; in Difflugia, Jennings ('16) found strains that differed in a number of characters (number and length of spines, diameter of shell, etc.); in Centropyxis, Root ('17) isolated strains differing in number of spines and the size of shell; in Arcella, Hegner ('20) found another protozoan that showed the same phenomena, the lines differing in spine number and diameter of shell within the same clone.

In comparison with the mass of experimental data in the protozoa, a relatively small amount of work has been done on forms reproducing parthenogenetically, although in the species where no reduction in the number of chromosomes occurs, the reproduction is as typically uniparental as in the cases where an organism reproduces by fission.

Kelly ('13) tried by selection to alter the relation of the third to the fourth antennal joint in a parthenogenetic aphid (Aphis rumicix), but the work was carried on for only two generations and no effect produced.

Agar ('14) worked with three parthenogenetic Cladocera, Simocephalus exspinosus, S. vetulus, and Daphnia obtusa, and a parthenogenetic aphid, Microsiphum antherinii. In both species of Simocephalus an attempt was made to increase the body length by selection, but the coefficient of correlation between individuals and their ancestors showed no diminution as the scale of ancestors was ascended, and in Daphnia also there was "no evidence of inheritance of interclonal variation." In Microsiphum antherinii, however, there was a diminution in the intensity of the correlation from the parental to the grandparental relation, which indicated that selection within the clone had been effective. In a discussion of this part of the work, Agar suggests that such a conclusion must be stated tentatively, since in all the cases the progeny are produced viviparously and have short life histories, so that there is a comparatively short time to eradicate the effects of intra-uterine life. Ewing ('14 a, b, '16) studied various characters of Aphis avenae Fab., and in the first ten generations attempted by selection to increase

or decrease the ratio of the third and fourth antennal segments. He concluded that "Selection from among extreme variants does not alter the mean as obtained for the strain without selection. The offspring of an extreme variant may show, not a reversion to the mean of the line of a strain, but a reversion which swings pendulum-like much beyond the mean . . . . only to be brought back to its former side of the mean-of-the-strain base line in the next generation." In the second paper this pendulum-like swinging of the mean for the offspring was tested through seven generations with the conclusion that "regression within a pure line of a parthenogenetic form does not follow Galton's law. . . . but . . . . is somewhat pendulum-like, swinging beyond the mean of the strain, or line." In the last paper extensive experiments carried on with the cornicles, the antennae, and the body length yielded results which pointed to the conclusion indicated in the earlier work, that selection in a parthenogenetic line had no lasting effect.

At the present time, then, experimental data on uniparental inheritance point in opposite directions, some work indicating that selection within a pure line is ineffective (Johannsen, Jennings, Ewing, and many botanists), other work, that selection is effective (Jennings, Middleton, Root, Hegner). There was some indication in Proales that an increase in length of life and in number of eggs produced had taken place during the time of cultivation in malted milk. A comparison of 100 organisms isolated in February, 1919, with 100 organisms isolated in June, 1919, shows an increase in average egg production from 12.79 to 23.43, with an increase in maximum production from 23 to 30. This increase in egg production was accompanied by an increase in individual range of life from 3 to 5 days to 2 to 7 days. Such increase in egg production and length of life suggested that by selection the maximum in both these might be increased still further. An experiment was therefore undertaken to test this. In this experiment in selection an attempt was made to eliminate variations in the conditions as much as possible; the food was prepared carefully from the same jar of malted milk, with water from the same spring throughout the

experiment, and all organisms after the fifth generation were kept in an electric constant temperature oven at 23° to 25°C. Since egg production and length of life are correlated as indicated above, the number of eggs produced rather than the length of life was decided upon as a more reliable measure of performance, and an attempt was made by selecting in each generation the individual producing the highest number of eggs to increase the maximum number of eggs produced by any individual beyond thirty, a number frequently obtained in individuals chosen at random.

The progenitor for this selection work was chosen at random from the progeny of a mother producing twenty-four eggs. The individual thus chosen produced twenty-seven eggs, designated as the first generation. All these eggs hatched, but two of the young organisms died before producing eggs. The minimum number of eggs produced by any of the twenty-five sister individuals in the first generation was 5, the maximum 28, the average 18.68.

A check-line was reared under the same experimental conditions and isolations made at the same time as in the selection experiment, but the progenitor for the next generation was chosen at random rather than by an inspection of the egg-depositing record. In the first generation the check-line deposited 18 eggs, a number slightly below the average, 18.68, for the selection line. The individual range for the length of life in the selection experiment was two to six days with an average of 4.36 days as compared with an length of life of four days in the check. During the first generation the selected line showed a much higher maximum and average number of eggs, and a greater average length of life than the non-selected line.

The further course of the experiment, through fifteen generations of selection, is shown in table 3. Throughout the experiment the individual producing the highest number of eggs was chosen to continue the selected lines, while for the control line an individual was taken at random.

As will be seen in table 3, fifteen generations of selection of the individual producing the highest number of eggs did not bring

about any clear increase either in the maximum number of eggs produced, the individual range of life, or the average length of life. In four generations the average length of life in the selected line is greater than the length of life in a line of individuals chosen at random. In three generations the average number of eggs produced by the selected line is greater than the number produced by an individual in a non-selected line. In all other cases

TABLE 3

A comparative table of the selected and non-selected lines of Proales, showing the numbers of eggs produced and the length of life

GENERATION	SELECTED INDIVIDUALS						NON-SELECTED INDIVIDUALS	
	Number of eggs produced			Length of life in days			Number of eggs produced	Length of life in days
	Minimum	Maximnm	Average	Minimum	Maximum	Average		
1	5	28	18.68	2	6	4.36	18.00	4
2	2	27	13.85	2	6	3.42	16.00	4
3	2	30	17.00	2	6	4.04	17.00	4
4	6	28	19.00	2	6	4.02	21.00	5
5	4	26	17.14	1	4	3.71	18.00	4
6	1	27	18.56	1	5	3.60	19.00	4
7	3	28	15.57	2	6	4.23	22.00	6
8	3	28	18.03	2	6	4.97	20.00	5
9	1	29	13.84	1	7	4.80	25.00	6
10	2	28	20.14	3	7	5.22	20.00	4
11	8	26	17.92	3	6	4.68	24.00	6
12	7	26	17.03	3	5	4.42	19.00	5
13	4	27	17.20	2	7	4.64	16.00	4
14	6	26	17.66	3	6	5.08	22.00	6
15	3	25	16.70	3	6	4.66	25.00	7

the selected line shows an average either equal to or below the numbers produced in a non-selected line. These results indicate that at this time selection for a greater number of eggs and a longer average life-cycle in Proales is ineffective. The results obtained from this selection work do not offer any explanation for the great variation in maximum and average length of life, in range of individual life-cycle, and in maximum and average egg production mentioned earlier as occurring between the

earlier and the later periods of the work. Such an increase may have been due to the greater degree of adjustment to the environment in the individuals constituting the race after cultivation for six months in malted milk.

THE EFFECT OF ALCOHOL IN RELATION TO INHERITANCE

Can the inherited characteristics of organisms be changed by environmental agents? Particularly, is it possible by poisons, by extremes of temperature, or by excessive concentration of salts in themselves non-toxic, so to injure germ cells that later generations will be weak, abnormal, or essentially different from their parents?

This phase of the general problem of the causes of variation in organisms has been attacked more extensively in mammals and birds than in any other members of the animal kingdom. The very interesting and important sociological significance of a limited part of the problem, namely, the effect of alcohol on human welfare, has resulted in the bringing together of a large mass of data, unscientifically collected and arranged for the most part, pointing in a general direction too familiar to require setting forth here. The larger mass of the experimental data on the same subject has been collected during the last few years on guinea-pigs and the domestic fowl. Stockard ('18), working with guinea-pigs, has studied for over seven years the behavior of these animals when subjected to the influence of alcohol. Not only has the effect of alcohol on the individual animal been studied, but also the effect of alcohol on the progeny when matings were made between alcoholized animals, between alcoholized animals and normal animals, and between the progeny of alcoholized animals and normal animals as far as the third generation. In regard to the effect of the alcohol on the individual animal the earlier work is summed up as showing "that the germ cells in either the male or the female mammal may be changed or affected by a chemical treatment administered to the body of the animal" (L 120). The progeny of animals thus treated "showed more or less marked deviation from the normal in many definitely measurable qualities, such as their mortality records, structural

appearance, nervous reactions, and ability to reproduce" (p. 120). When the progeny of alcoholized animals, themselves not subjected to the treatment, were mated the data were such that "it may be concluded that animals as far as three generations removed from the direct alcohol treatment are still differentiated from the control in regard to the weight of the litters in which they are born, the tendency of the matings to result in failure . . . ." (p. 164). In general, then, the alcoholization of guinea-pigs produces some change in the germ cells which is still evident in the third generation of non-treated progeny, even when normal germplasm is thrown into the hereditary stream at each mating.

In the domestic fowl, Pearl ('17 a, b, c,) has used the same general method of treatment, in this case treating the birds for only one hour per day. In appearance "the treated animals themselves are not conspicuously worse or better than their untreated control sisters and brothers" (p. 185). The capacity to reproduce was not influenced since "neither the total amount nor the distribution of egg production were significantly different in the treated birds from what they were in the controls" (p. 186). But "the proportion of fertile eggs . . . . was materially reduced in the matings in which one or both individuals had been treated" (p. 294). At the close of the account of the experiment the conclusion was reached that "There is no evidence from these experiments that the treatment of individual fowls with ethyl alcohol had any deleterious effect upon those germ cells which form zygotes. The treatment rendered many germ cells incapable of forming zygotes at all, but those which did form zygotes had plainly not been injured in any way" (p. 295).

Thus among the higher organisms the evidence points in opposite directions, that secured from mammals indicating the persistence of the influence of the environmental agent as far as the third generation of the untreated progeny, that secured from birds not indicating that there is a change produced in the germ cells by the subjection to alcohol. Among the lower organisms Whitney ('12) has studied the effect of alcohol on the rotifer Hydatina senta. He reared the organisms in culture fluid plus $\frac{1}{4}$, $\frac{1}{2}$, and 1 per cent ethyl alcohol for twenty-eight

generations, starting each new generation with a daughter from an alcoholized mother. At the end of the experiment progeny of these alcoholized lines were returned to normal culture solutions to test the retention of the influence of the alcohol. In regard to the production of eggs, it was found that "The rate of reproduction was lower in the alcoholic strains than in the control and it was proportionally lowered according to the amount of alcohol used." But after the return of the progeny to normal culture solutions it appeared that "The weaknesses developed by the parental use of alcohol are partially eliminated in the first generation after the alcohol has been removed, and practically completely eliminated at the end of the second generation after the alcohol has been removed." In so far as such a comparison can be made, the rotifer Hydatina senta reacts to alcohol in much the same way as the domestic fowl.

The question of the effect of alcohol on germ cells is of such importance that it seemed worth while to test it again as thoroughly as possible in another of the lower organisms. Proales seemed particularly favorable for this work, since it will thrive in a medium of comparatively definite composition, passes through the life-cycle in a short time, and produces a large number of eggs with a high percentage of viability. In preliminary study it was found that individuals of Proales subjected to the fumes of ethyl alcohol in solutions of varying percentages showed an effect equal to that on individuals reared in culture solutions to which an equal per cent of alcohol had been added directly. The same thing has been found to be true for infusoria by Estabrook ('10), working on Paramecium, and in many other experiments carried out in this laboratory. Throughout the experiment with Proales the organisms have been isolated in normal $\frac{1}{16}$ per cent malted milk and placed on supports in closed Stender dishes. In the bottom of each culture dish sufficient absolute alcohol was added to 100 cc. tap-water to give the percentage indicated in the particular experiment cited. These alcohol solutions were changed each day, the adult organisms transferred to fresh culture fluid, and if individual data were to be secured on the eggs, they were transferred to fresh culture fluid also.

In September, 1919, 100 individuals were chosen at random from the mass cultures established in August, these being originally all descendants of a single individual. These were reared under normal conditions until they had each deposited three or more eggs. Three eggs from each of these 100 organisms were used to start the experiment; one from each individual was placed in a culture dish with 1 per cent alcohol in the solution used in the bottom, another in a dish with 2 per cent alcohol, and a third in a dish with tap-water to serve as a check for the alcohol lines. The cultures were examined daily, the alcohol solutions changed, and the adults transferred to fresh food. At the end of the first week 100 organisms were isolated from each main line and the length of life and the egg production determined in each. (See table 4 for a diagrammatic scheme of the alcohol experiment.) At the end of the first week the range in egg production for the 100 individuals from the 1 per cent alcohol line was 1 to 7, with an average of 2.85, while the range in the length of life was 2 to 6 days, with an average of 3.88 days; in the line reared subjected to 2 per cent alcohol the range in egg production was 1 to 6, with an average of 1.84; the range of individual life was 2 to 5 days, with an average length of life of 4.19 days; in the check line the egg production ranged from 6 to 30, with an average of 20.86, while the range in the length of life was 3 to 5 days, with an average of 4.78 days. At the end of the first week individuals from each of the alcohol lines showed a decided reduction in the power to deposit eggs; those subjected to 1 per cent alcohol depositing an average of 2.85 eggs, and those subjected to 2 per cent an average of 1.84 eggs, as compared with an average deposit of 20.86 eggs in individuals reared under normal conditions. The average length of life did not show as great a reduction; subjected to 1 per cent alcohol, the individuals lived an average of 3.88 days; to 2 per cent, an average of 4.19 days, while those under normal conditions had an average length of life of 4.78 days. Thus there is a marked reduction in the egg-laying capacity, unaccompanied by so great a reduction in the average length of life, early in the history of a line reared in alcohol. The viability of the eggs produced in these per-

centages of alcohol was so low (less than 25 per cent) that it soon became evident that it would be impossible to continue the lines indefinitely. Hence the line in 2 per cent alcohol was discontinued and the progeny produced in 1 per cent alcohol divided into two lots, one to be subjected to ¼ per cent alcohol, the other to ½ per cent. The organisms were reared for two weeks over these percentages of alcohol; isolation of eggs for new generations were made whenever the age of the majority of adults

TABLE 4

Diagrammatic scheme of alcohol experiment

```
                        1" wk.          2" wk.   3,4" wks. 5" wk.  6,7" wks.        8"

                       2% ethyl alcohol.
                          1 wk.
Feb.1919-Sept.1919.  Malted milk  ──────────→ Malted milk. ───→ Malted milk. ───→
    Malted milk.          1 wk.                  2 wks.            2 wks.
                      1% ethyl alcohol.      ↘ ½% ethyl alcohol.→½% alcohol.  ───→
                          1 wk.                  2 wks.            2 wks.
                                             ↘ ¼% ethyl alcohol.→¼% alcohol.  ───→
                                                 2 wks.            2 wks.


  9,10" wks. 11" wk.   12,13" wks. 14" wk.15,16" wks. 17" wk.  18,19" wks. 20" wk

Malted milk. ──────→ Malted milk. ─────→ Malted milk. ──────→ Malted milk. ───→
   2 wks.               2 wks.              2 wks.               2 wks.
½% alcohol.  ──────→ ½% alcohol.  ─────→ ½% alcohol.  ──────→ ½% alcohol.  ───→
   2 wks.               2 wks.              2 wks.               2 wks.
¼% alcohol.  ──────→ ¼% alcohol.  ─────→ ¼% alcohol.  ──────→ ¼% alcohol.  ───→
   2 wks.               2 wks.          ↘   2 wks.               2 wks.
                                        ↘ 1% alcohol.
                                              2 wks.
                                          2% alcohol.
                                              2 wks.


  21,22" wks. 23"wk.    24" wk.    25" wk.  26" wk.    27" wk.
Malted milk. ──────→ Malted milk. ──────→ Malted milk.
  2 wks.                1 generation.     1 generation.
½% alcohol.  ──────→ Malted milk. ──────→ Malted milk.
  2 wks.                1 generation.     1 generation.
¼% alcohol.  ──────→ Malted milk. ──────→ Malted milk.
  2 wks.                1 generation.     1 generation.
```

seemed to warrant. At the end of this time (five weeks) 100 individuals from each line were isolated and the egg production and length of life determined. In the ¼ per cent alcohol the range of egg production was from 4 to 22, with an average of 14.25; the range of life from 3 to 7 days, with an average length of life of 5.83 days in the ½ per cent alcohol the range in egg production was from 1 to 10, with an average of 3.98; the range of of life from 4 to 7 days, with an average of 6.09 days; in the controls the egg production ranged from 14 to 30, with an average of 14.10 eggs; the length of life from 3 to 7 days, with an average

of 6.17 days. When these percentages of alcohol are employed, the reduction in the number of eggs deposited is not so marked as in higher percentages, but the difference between the alcohol lines and the line reared under normal conditions is still great. There is little reduction in the average length of life in either percentage of alcohol, just as was the case where 1 per cent and 2 per cent were used.

The alcohol experiment was carried on for twenty-seven weeks in the way just indicated; that is, 100 individuals each subjected to ¼ and ½ per cent alcohol solutions, and 100 controls, were allowed to reproduce for a period of two weeks, when isolation of 100 specimens was made from each line and the egg deposit and length of life for all the individuals determined under each of the three conditions; reproduction continued for another two-week period, then another isolation made, etc., until the end of the twenty-third week. At this time the alcohol cultures were discontinued and progeny from both alcohol lines were returned to malted-milk solution without alcohol. In this way it was determined whether the effects of ¼ and ½ per cent alcohol, which had been acting continuously on the progenitors of both lines for twenty-one weeks, had any lasting effect when the progeny were returned to malted milk. At the beginning of the fifteenth week progeny of the line reared in ½ per cent alcohol were isolated and reared in 1 per cent and 1½ per cent alcohol for a period of two weeks and an isolation made at the end of this time to determine if under the continuous action of ½ per cent alcohol the organism had developed any degree of resistance to higher percentages.

The effect of the alcohol upon the organisms themselves was marked only in the higher percentages. In many cases in these percentages the increase in size, which usually continues until near the close of the life-cycle, never took place and the individuals were thin and attenuated; movement in many cases was reduced even in the young individual, and the adults became very sluggish. In the experiments where a low percentage of alcohol (¼, ½ per cent) was employed, the treated organisms resembled the untreated checks except in the number of eggs deposited.

An isolation made at the end of the tenth week showed that in the $\frac{1}{4}$ per cent alcohol line the range of egg production was from 1 to 19, with an average of 11.07. This is slightly lower than the results in the fifth week, where the range was from 4 to 22, with an average of 11.25. In the eleventh week the range of life was from 3 to 8 days, with an average of 6.46. In the fifth week the range of life had been from 3 to 7 days, with an average of 5.83 days. In the eleventh week in $\frac{1}{4}$ per cent alcohol the average and maximum production of eggs had fallen below that in the fifth week, but the maximum length of life at this time was the greatest attained by any individual throughout the entire study. A greater length of life under treatment with alcohol was noted by Stockard in one of his guinea-pigs, which lived seven years—an unusual time in that organism. Individuals subjected to $\frac{1}{2}$ per cent alcohol showed at this time a range in the egg deposit of 1 to 10, with an average of 5; this was the same as the range in the fifth week, but at that time the average was only 3.98. The range of life for this period was from 3 to 7 days, with an average of 5.70 days, as compared with a range of 4 to 7 days and an average of 6.09 days at five weeks. In malted milk under normal conditions the range of egg production at the eleventh week was 8 to 29, with an average of 19.70, while the range in life was 3 to 7 days, with an average of 6.48 days. At this isolation, just as in the previous one, there is a decided reduction in the number of eggs produced in both the alcohol lines and very little reduction in the average life-period.

At the beginning of the fifteenth week two new isolations of 100 individuals each were made from the $\frac{1}{2}$ per cent alcohol line; one of these was reared in 1 per cent alcohol; the other in $1\frac{1}{2}$ per cent. At the beginning of the seventeenth week isolations were made for the determination of the egg production and the length of life from the five lines, malted milk, $\frac{1}{4}$ per cent, $\frac{1}{2}$ per cent, 1 per cent, $1\frac{1}{2}$ per cent alcohol. At this time the line in $\frac{1}{4}$ per cent alcohol showed a range in egg production of 4 to 22, with an average of 10.80; a range in the length of life of 3 to 7 days, with an average of 5.88 days. The line in $\frac{1}{2}$ per cent alcohol showed an egg production ranging from 1 to 10, with an

average of 4.55, and a range of length of life of 2 to 7 days, with an average of 6.52 days. The line previously reared in $\frac{1}{2}$ per cent alcohol and transferred to 1 per cent only two weeks previous to this isolation showed a range in egg production from 1 to 7, with an average of 2.98, a range in length of life from 3 to 7 days, with an average of 5.22 days. The line recently transferred to $1\frac{1}{2}$ per cent alcohol showed a range in egg production of 1 to 6, with an average of 2, and a range in length of life of 2 to 7 days, with an average of 4.91 days. The line reared continuously in malted milk under normal culture conditions showed a range for egg production of 1 to 28, with an average of 19.26, and a range in the length of life of 3 to 7 days, with an average of 5.81 days.

In general during the twenty-one weeks in which individuals of a line of Proales were subjected continuously to the fumes of $\frac{1}{4}$ and $\frac{1}{2}$ per cent ethyl alcohol the maximum and average number of eggs produced was reduced in proportion to the percentage of alcohol used, but the average length of life was very little influenced. Table 5 gives a summary of the range and average egg production and the range and average length of life of all the individuals studied throughout the experiment. In neither of the lines subjected to $\frac{1}{4}$ and $\frac{1}{2}$ per cent alcohol is there a continual decrease in the average of either character studied throughout the successive generation.

Tests for inheritance of the effect of alcohol. At the beginning of the twenty-fourth week both alcohol lines were discontinued and their progeny returned to malted milk only. At the end of the first week in normal conditions (twenty-fifth week of the experiment) isolations of the second generation individuals were made for determining the egg production and average length of life. In the line descended from the progeny of the $\frac{1}{4}$ per cent alcohol group the range of egg production was from 9 to 27, with an average of 17.77, as compared with a range of 3 to 23, with an average of 12.33 eggs in the last generation in $\frac{1}{4}$ per cent alcohol. The range of life at this time was 3 to 7 days, with an average of 5.58 days, differing little from the range and average in the last isolation in $\frac{1}{4}$ per cent alcohol. In the line descended from individuals reared in $\frac{1}{2}$ per cent alcohol the range in egg produc-

tion was from 5 to 25, with an average of 13.57, as compared with a range of 1 to 11 and an average of 3.48 in the last generation in alcohol. The average length of life, 5.70 days, had increased very little over the average of 5.62 days in the last isolation. Individuals reared continually in malted milk showed at this time a range in egg production from 4 to 29, with an average of 18.56, and a range in length of life from 2 to 7 days, with an average of 5.56 (table 4).

TABLE 5

A comparison of the egg production and length of life of all isolations made of lines subjected to ¼ per cent and ½ per cent alcohol and controls

NUMBER OF WEEK AT WHICH ISOLATION WAS MADE	LINE IN ¼ PER CENT ALCOHOL				LINE IN ½ PER CENT ALCOHOL				CONTROLS			
	Egg production		Length of life		Egg production		Length of life		Egg production		Length of life	
	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average
5th	4–22	14.25	3–7	5.83	1–10	3.98	4–7	6.09	4–30	24.10	3–7	6.17
6th	3–20	12.63	2–7	5.60	1–10	4.44	2–6	5.08	4–29	20.95	2–7	5.16
11th	1–19	11.07	3–8	6.46	1–10	5.00	3–7	5.70	8–29	19.70	3–7	6.48
14th	4–22	12.67	3–7	5.10	1–11	4.99	3–6	5.16	5–28	15.84	3–7	5.99
17th	4–22	10.80	3–7	5.88	1–10	4.55	2–7	5.52	4–28	19.26	3–7	5.81
20th	4–23	14.73	3–7	5.95	1–11	4.39	2–7	5.45	5–28	20.59	3–7	5.80
23rd	3–23	12.33	2–7	5.95	1–11	3.48	2–7	5.62	5–28	18.88	2–7	4.75
*												
25th	9–27	17.77	3–7	5.58	5.25	13.57	2–7	5.70	4–29	18.56	2–7	5.56
27th	12–30	21.80	3–7	5.98	8–29	19.92	3–7	5.37	11–30	19.50	4–7	6.06

* Indicates time when alcohol cultures were transferred to malted milk.

Thus, individuals of the second generation after the return to normal conditions showed a marked increase in egg production; in other words, only a partial retention of the influence of the alcohol; and the averages for the generation whose ancestors were subjected to alcohol approach those individuals continually reared in malted milk.

Isolations of the third generation made under the same conditions as have just been described at the beginning of the twenty-seventh week, show an average egg production of 21.80 for descendants of individuals subjected to ¼ per cent alcohol, and 19.92 for those subjected to ½ per cent, as compared with an average

of 19.50 for checks reared continuously in malted milk. At this time, after three generations spent in malted milk, all the effects of alcohol upon egg production has been lost.

To summarize: In Proales decipiens individuals of a line subjected to the fumes of $\frac{1}{4}$ and $\frac{1}{2}$ per cent ethyl alcohol continuously for nineteen weeks show a decided reduction in egg production while under the influence of the alcohol, but their progeny, returned to normal conditions, regain the normal egg-producing power after the third generation.

SUMMARY

This paper is an account of the normal life-cycle of Proales decipiens, with experimental studies of the production of males, of the effects of selection during parthenogenetic reproduction, and of the effects of alcohol on inherited characteristics.

1. Statistics are given as to the length of life, the rate of reproduction, the number and kind of eggs deposited, with study of the variations in these matters. The animal lives about a week, then dies with characteristic symptoms of senility. During its life it produces several eggs per day, the number increasing to a maximum, then decreasing with the onset of old age.

2. Reproduction by parthenogenesis for about 250 generations gave no indication of reduction of vigor in the race in any respect.

3. During this period, no males appeared. Alteration of the environment by changes in the nature and concentration of the food, by changes in the temperature at which cultures were reared, and changes in the chemical constitution of the medium were not accompanied by the appearance of the male form. So far as known, the species may be quite without males.

4. An attempt to increase the egg deposit and average length of life through artificial selection carried on for three months, in fifteen generations, was without avail, placing this organism in the list with other parthenogenetic forms in which selection is ineffective.

5. Treatment with ethyl alcohol in a concentration of $\frac{1}{4}$ and $\frac{1}{2}$ per cent for twenty weeks reduced the number of eggs produced from an average of 15 to 24 in normal malted milk to an average

of 10 to 14 in $\frac{1}{4}$ per cent alcohol, and 3 to 5 in $\frac{1}{2}$ per cent alcohol, although the length of life was little influenced.

6. The reduction in egg deposit brought about by alcohol was not retained beyond the third generation of descendants restored to normal conditions of culture.

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SUMMARY

With a new apparatus the mechanism of breathing of Dytiscus marginalis L. is studied. The movements of a droplet of petroleum are observed when the animal is allowed to breathe in a space which is closed by this droplet. It appears that the first observable movement is an expiration, which fact is in contradiction with previous statements of other authors. A theory is given to explain this fact and in several ways the verification of this theory is tried. With a new apparatus the air is studied after the animal is allowed to breathe in it for some time. The fact reported by Ege and Kreuger that the O_2 in the air-store is consumed during the animal's stay under water was proved again. The biological importance of the oxygen diffusion into the air-store is discussed from the physico-chemical standpoint and treated mathematically.

Finally, the relative importance of the gases in the water and in the air for the animal's behavior is studied. It appears that though the animal has become emancipated to a certain extent from its original environment, it is still more dependent on the air than on the gas content of the water.

Thanks are due to Prof. Dr. Withrow Morse who tried to make my English readable even for the English reader—and I trust he succeeded—and corrected the many mistakes.

MORGANTOWN, W. VA., U. S. A., January 6, 1921

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Resumen por el autor, William H. Cole.

La transplantación de la piel en los renacuajos de rana, con especial mención del ajustamiento de los injertos sobre los ojos y de la especificidad local del tegumento.

Los injertos de piel sobre los ojos de los renacuajos (Rana catesbiana y R. clamitans) son reabsorbidos de un modo que tiende a exponer los ojos restaurando de este modo la visión. Los injertos de piel de la cola sobre hemisferios de vidrio o celoidina son reabsorbidos hasta dejar libres dichos hemisferios, si bien no existe reabsorción cuando se depositan los injertos sobre placas delgadas de dichas substancias. Los injertos de piel del dorso sobre los ojos o los hemisferios no son reabsorbidos. No existe, por consiguiente, regulación funcional de los injertos de la piel sobre los ojos de los renacuajos. La absorción de los injertos de piel de la cola es enteramente una reacción mecánica a la tensión producida por la curvatura del ojo. Los injertos de piel del dorso, a consecuencia de su estructura más compacta, no reacionan a dicha tensión.

Los injertos de piel de la cola, dorso y abdomen en varias partes del cuerpo indican que existe una especificidad local del tegumento de los renacuajos de la rana y que la piel se autodiferencia cuando se transplanta en nuevas regiones de un mismo animal. La adquisición de melanóforos por los trozos de piel blanca del abdomen autotransplantados es principalmente el resultado de la formación de pigmento en las células epiteliales in situ. En los homoio-injertos es principalmente el resultado de la emigración epidérmica que transporta melanóforos desde la piel vecina. Las heridas extensas de la conjuntiva resultan en una capa regenerada que contiene melanóforos epidérmicos y dérmicos persistentes y xantoleucóforos dérmicos. La temperatura baja, la oscuridad, una solución de cloretona al 0.I por ciento, la anoxemia y el bajo metabolismo de un animal moribundo producen expansión de los melanóforos dérmicos. Su contracción es el resultado de la temperatura elevada, la luz y el regreso a las condiciones ambientes normales.

Translation by José F. Nonides
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THE TRANSPLANTATION OF SKIN IN FROG TADPOLES, WITH SPECIAL REFERENCE TO THE ADJUSTMENT OF GRAFTS OVER EYES, AND TO THE LOCAL SPECIFICITY OF INTEGUMENT

WILLIAM H. COLE

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TWO TEXT FIGURES AND FOUR PLATES (TWENTY-TWO FIGURES)

CONTENTS

INTRODUCTION

The fact of dependent differentiation has been observed in the development of several animals. It has been demonstrated that certain cells of the early embryos will differentiate into the anlage of an organ, providing certain other cells are present to originate the stimulus for such a change. In some cases by appropriate operations, a third group of cells from a different region of the embryo may replace the first group, and will show the same kind of differentiation. Such 'totipotence' of embryonic cells may persist to a certain degree in the adult state of lower invertebrates, as shown, for example, by the remarkable regenerative power of coelenterates and worms. But in the higher vertebrates totipotence is lost completely, though at different stages of development in different animals.

During the past twenty years the organogeny of the vertebrate eye has been investigated extensively to discover any correlation that may exist between the development of one part and that of another. In these studies certain parts have been injured, removed, transplanted, or replaced by strange parts, in attempts to solve the problem. The researches of Spemann ('01, '03, '07, '12), Lewis ('04, '05), King ('05), Le Cron ('07), Stockard ('10), and Fessler ('20) are the most noteworthy. In general it has been concluded that a certain amount of correlation between the development of the parts of the eye exists, but that also some of the parts have become, or are becoming, self-differentiating. For example, the formation of the lens is stimulated and aided by the optic cup, although under certain conditions it may develop without the influence of the optic cup (Stockard, '10). A second example is furnished by the appearance of transparency in the ectoderm over the eye. In normal development the ectoderm over the eye loses its pigment, is thinned, and becomes transparent. If ectoderm from a strange region of the embryo replaces the normal ectoderm, the former becomes similarly transparent. If, however, the eye is removed, no transparency results. These discoveries have enlivened the age-old discussion concerning the relation between the form and function of an organ. Modern biologists may be divided into two groups, according to their

interpretation of this relation. It is held, on the one hand, that function is causal to form and structure, while on the other, that it is resultant.

In line with the studies on the production of transparency in the ectoderm over the young eye, it was suggested to the writer by Prof. H. W. Rand that the experimental inhibition of vision by grafting opaque skin over the eyes of frog tadpoles, long after the eyes had been fully formed, might contribute evidence as to any interacting influences between the graft and the eye. Possible results of such an experiment are, first, that the graft may become transparent by a thinning process and by a loss of its pigment cells, thus repeating the embryological history of the ectoderm over the eye. This would indicate that there is a direct relation between the visual function and the production of transparency. Secondly, the transplant may remain unchanged, inhibiting vision permanently. Such a result would show that the skin had lost its power of becoming transparent or that the eye exerted no influence upon the graft. Thirdly, the transplant may be completely absorbed or thrown off, thus restoring vision and strengthening the causal-function theory. In my early experiments, which consisted of grafting tail skin over the eyes of frog tadpoles, the first and third possibilities were not realized; the second one sometimes was, while the majority of the transplants exhibited an entirely unexpected kind of adjustment or regulation. This adjustment consisted of the absorption of part of the graft in such a way that light could reach the eye directly. When this was accomplished, absorption ceased. This discovery suggested that the function of the eye might be causal to the adjustment process. Since no satisfactory explanation could be formulated from the results, further experiments were planned to answer the question, Is the absorption process really initiated and controlled by the interruption of vision? During the greater part of the investigation this question could not be definitely answered in the negative, and much evidence pointed to its affirmation. Finally, from the results of check experiments, in which 'artificial eyes' were used beneath the grafts in other parts of the body, proof was obtained that the visual function does not

control the process, and the mystery of the earlier cases disappeared. Partial absorption of the graft, which occurred in so many cases, was undoubtedly caused by a mechanical stimulus, probably a tension.

During the study of the pseudoregulation which was observed in transplants taken from the tail region only, the problem of a possible local specificity of integument was presented. With this problem in mind, the experiments were continued, using skin from three different regions of the tadpole, namely, posterior part of tail, the belly, and the back. The results obtained can be interpreted only by assuming that in the integument of those regions there are specific substances which retain their individuality when transplanted to another region and which determine the future history of the transplant. The grafts which were taken from the belly region were white, due to the absence of melanophores. After transplantation to the dark back region such grafts acquire melanophores. An attempt was made to discover the method of such acquisition. Autotransplants and homoiotransplants of belly skin on the back region were studied, and their behavior led to the conclusion that the source of the graft determines the method of melanophore appearance. Homoiotransplants are pigmented chiefly by migration of epidermal melanophores from the surrounding skin. Autotransplants are pigmented chiefly by formation of melanophores in situ. Finally, the pigmentation of the conjunctiva, which may be produced experimentally by an injury to that tissue, was investigated. The description of such experiments will be found in the last section of this paper.

It is not the purpose here to review the extensive literature on the transplantation of tissues and organs. Since the time of John Hunter, about the middle of the eighteenth century, zoologists and medical men have transplanted all kinds of tissues and organs under many different conditions and on different species of animals. Korschelt ('07), Schöne ('12), and especially Barfurth ('14) have arranged excellent bibliographies with discussions of the important researches on this subject. For the earliest works, the lists given by Bert ('66) and Reverdin ('92) are

inclusive. Throughout the following pages many of the terms and ideas given by Loeb ('20 a, '21) and his co-workrs are applied in the discussion of my experiments. Other references to the literature are specifically quoted in the text, and a list is appended at the end of the paper.

I wish to express my grateful acknowledgment to Prof. H. W. Rand for his kindly interest and helpful criticism during the progress of the experiments. The work was begun in the fall of 1915 at Harvard University and continued until the following summer. During the next two years it was carried on at the Pennsylvania State College, until interrupted, in the winter of 1917, by the war. In October, 1920, the experiments were resumed at Harvard and completed in the spring of 1921. I wish also to thank Prof. E. L. Mark, Director of the Zoölogical Laboratories at Harvard, for the privileges of the laboratory and for many courtesies extended to me.

MATERIAL AND METHODS

Because of the ease in handling the animals and in making transplantations, frog tadpoles were selected for the experiments. They were of two species, Rana clamitans and R. catesbeiana, and were obtained from three different sources, viz., from ponds either near Cambridge, Massachusetts, or State College, Pennsylvania, and from Boston dealers in animals, who secured their supply from 'Illinois.' The tadpoles were collected from the ponds during the fall and brought into the laboratory or purchased from the dealers during the winter months. In length they varied from 20 to 100 mm., representing different ages and stages of development. No significant differences in the histories of the grafts, excepting white grafts on black regions, could be correlated with differences in species, size, or age.

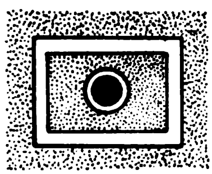
A large balanced aquarium with a mud bottom provided the stock animals an almost natural environment. The tadpoles being experimented on were kept singly in small battery-jar aquaria. Attempts to feed the animals were mostly unsuccessful. Earthworms, cooked beef, boiled egg yolk and clam meat, as well as

decaying animal matter, were offered without success. The actual food supply seemed to come from the algae, plants and mud in the jars. The absence of the vigorous feeding reactions shown by young tadpoles in the spring and summer was very marked. It was concluded that frog tadpoles of these species must have a lowered rate of metabolism during the winter months. In their natural habitat, no doubt, such a lowered metabolic rate occurs, due partly to the continued low temperature of the water. However, aside from this, the animals appeared normal in every way and responded to the usual stimuli. Many of them were kept in the small jars seven and eight months, a few of them undergoing metamorphosis. Except in some of the experiments to be noted later, the jars were kept on a table in diffuse daylight.

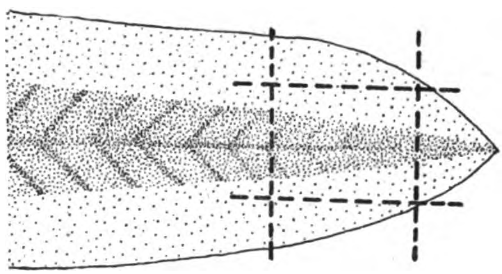
The method of procedure in transplanting integument was as follows. The animals were placed in 0.1 per cent aqueous solution of chloretone (trichlortertiarybutyl alcohol; Parke, Davis & Co.), which anaesthetized them sufficiently for the operation in from six to fifteen minutes. The minimal time for anaesthetization is determined principally by two factors: size of the animal and the temperature of the solution. As the temperature increases, the time decreases; but as the size of the animal increases, the time increases. A square or other rectangular incision with a perimeter of about 24 mm., was made in the integument where the transplant was to be placed. In some cases all of the integument within the incision was removed; in others the integument was left within the incision. When the graft was placed over the eye, the conjunctiva lay within the incision, as shown in text figure A. The transplant consisted of integument taken from one of three different regions of the same animal (autotransplantations), or from a different animal not closely related (homoiotransplantations). The three regions were: posterior part of the tail, middle of the back, and middle of the belly. In every case it was cut so as to fit as nearly as possible the incision to which it was transplanted. The grafts taken from the tail consisted of one-half the thickness of that structure, containing integument, the muscle layer and the notochord. Since the transplant was taken from near the base of the caudal fin,

the amount of muscle tissue included was very small. Text figure B illustrates the shape of such a graft and the place from which it was taken.

After the transplant was fitted to the wound the animal, placed on a concave support, was lowered into a small battery jar, containing enough 0.04 per cent chloretone solution to cover the tail, ventral body region, and the mouth, while the wound with its graft was not covered. The tadpoles were then kept for two hours under these conditions, during which time the transplant established union with the edges of the surrounding



A



B

Text-figure A Diagram of the incision made in the integument around the eye. A narrow band of skin was removed, leaving the conjunctiva and some of the surrounding skin intact. The graft was then fitted to the incision. $\times$ 4.

Text-figure B Diagram of the posterior tail region from which the tail-skin grafts were taken. Dotted lines indicate the limits of such a graft. $\times$ 4.

integument. It was necessary to replace the chloretone solution by water whenever the respiratory rate became too low. This method of allowing the graft to unite with the surrounding normal integument, depending, as it did, on the coagulated serum and blood to hold the transplant in place, has obvious advantages over suturing or bandaging methods. At the end of two hours, the animals were placed in fresh tap-water to recover from the anaesthetic. Observations made daily thereafter consisted of recording any noticeable changes of form, position, pigmentation, and proliferation of the transplant and making sketches or photographs. For these purposes a glass cell (75 mm. long, 20 mm. wide, 25 mm. deep) containing water or chloretone solution

was used, the observations being made with a Spencer binocular microscope.

The following series of experiments were performed:

1. LN series (64 cases): Transplants over normal eyes of animals kept in the light.
2. DN series (16 cases): Transplants over normal eyes of animals kept in darkness.
3. LO series (13 cases): Transplants over eyes whose optic nerves had been cut; or over the sockets after the eyes had been removed. Animals kept in the light.
4. LB series (71 cases): Transplants on the body region of animals kept in the light.
5. LBE series (15 cases): Transplants over 'artificial eyes' on the body region of animals kept in the light.
6. DBE series (9 cases): Transplants like the LBE series; the animals kept in darkness.
7. LT series (13 cases): Transplants on the tail region of animals kept in the light.

The source of the transplants varied in the individual cases, as will be seen by referring to the tables which follow.

GENERAL OBSERVATIONS

1. Pigmentation

Among the stock animals there was very little difference in pigmentation of the integument from day to day. All of them were brown, varying from a light to a very dark shade. The animals used in the experiments, however, often showed great differences, ranging from complete expansion to complete contraction of the melanophores, depending on the conditions of their environment. To investigate the causes of such changes in pigmentation, tadpoles were exposed to varying conditions of temperature, illumination, and oxygen supply. It was found that, independently of ordinary illumination, a low temperature causes an expansion of the melanophores. Animals whose melanophores were maximally contracted were put into water at a temperature of 0°C., and exposed to diffuse daylight. After

twenty minutes the melanophores began to expand very slowly. At the end of two hours they exhibited about one-third of the expansion of which they are capable (fig. 20). During the next thirty-six to forty-eight hours, according to individual variations, expansion continued at a uniformly slow rate until the maximum was reached (fig. 19). In this condition the melanophores form an intricate network of processes, in which it is very difficult to distinguish the limits of the individual cells. During the course of expansion the color of the animal as seen by the unaided eye changes from a gray-green to a dark brown. The color of the pigment granules seen through the microscope is brown. At such a low temperature the tadpoles become very sluggish, being slightly anaesthetized. Brooks ('18) observed a similar reaction in the adult frog at a temperature of 5°C. When replaced in water at room temperature (20°C.), the reverse process takes place at the same rate; at the end of forty-eight hours, the color of the animal is again gray-green, the melanophores now appearing as single rounded masses typical of the contracted phase.

As might be expected from the reactions of other animals, the melanophores of the frog tadpole contract at high temperatures. Animals with maximally expanded melanophores were put into water at 35°C. and kept in complete darkness. After an exposure of from thirty-six to forty-eight hours, the melanophores were all contracted (fig. 21). Replaced in darkness at room temperature, expansion followed.

Light acts on the melanophores of frog tadpoles as it does on those of many other Amphibia, by causing their contraction. A good review of the literature of pigmentation in Amphibia is given by Dawson ('20). He found that in Necturus the melanophores expand in light and contract in darkness. This agrees with the 'primary reaction' of expansion due to light reported by Hooker ('14 a) for frog tadpoles. The 'secondary reaction,' after prolonged exposure, was a contraction. In my experiments light regularly caused contraction of the dermal melanophores. The primary reaction was not observed. Animals with completely expanded melanophores subjected to diffuse daylight on an indifferent background become light colored, and an examina-

tion of the melanophores shows them to be contracted. This condition, although beginning within an hour from the time light begins to act, requires about two days for completion. On a black background, however, in diffuse daylight, the melanophores will remain expanded, but in direct sunlight on a black background, they will contract. The absence of light produces expansion. Animals with contracted melanophores were put into complete darkness at room temperature. The course of the expansion which followed was almost identical with that in the temperature tests. At the end of two days the animals were dark brown, due to the close network of the melanophore processes. When replaced in diffuse daylight, the former condition was restored by the end of forty-eight hours.

It was further found that distilled water, boiled water, or a 0.1 per cent solution of chloretone, all at room temperature and in diffuse daylight, caused rapid expansion of the melanophores, the maximal amount being obtained in about one-half hour. Contraction is produced when the animals are replaced in tap-water in the light. In other words, expansion of the melanophores of the frog tadpole may be produced by anoxemia—a condition which is the reverse of that existing in the brook trout, as described by Lowe ('17). One other condition inducing expansion, observed frequently, is that of approaching death. Animals in a moribund state always show expanded melanophores, regardless of temperature and illumination. Such dark animals usually die after several days of subnormal behavior, although they may recover and the melanophores return to their previous state of partial contraction.

Responses of these pigment cells to temperature, chloretone, oxygen deficiency, and approaching death strengthen the hypothesis that expansion is caused by a decrease, and contraction by an increase in the metabolic rate of the whole animal. It may be assumed that, at the normal rate of the various chemical reactions going on in the body, certain substances specific for melanophores are produced in the proper amount to preserve the normal state of the pigment cells, whether this be a fully or only partly contracted phase. Any change in the rate of these reactions

would cause a change in the amount of the specific substances produced, and this, in turn, would cause expansion or contraction of the melanophores according to conditions. Redfield ('18) demonstrated that in the horned toad adrenin is the specific substance for the pigment cells. The effect of light is not so clearly explained by this assumption. However, it is commonly true that light acts on animals as a stimulant, causing an increase in metabolism, and that the absence of light acts like a narcotic. Assuming this for the frog tadpole, which is known to be photokinetic (Cole, '17; Obreshkove, '20), the hypothesis is supported by the facts of the experiments.

2. *Description of the healing process*

The removal of integument from any part of the tadpole's body initiates a stimulus which forces the epidermal cells surrounding the wound to migrate over it centripetally until a new covering is formed. This layer is at first only one cell thick, but further migration from the edges deepens it to several cells. As they move over the wound they carry along epidermal melanophores. Further increase in thickness of the new layer results from mitoses, which continue at a rate slightly above normal, until the original condition is closely approximated.

The rapid movement of epithelial cells over a fresh wound was first observed in the cornea of the frog by Peters ('85) and later in the salamander by Barfurth ('91) and again in the frog by Loeb ('98) and by Loeb and Strong ('04), who thought that it was caused by a tension "either previously existing or called into play by the wound." Such migration of epithelial cells has been seen in many different animals, and in tissue cultures it is a common phenomenon. In commenting on the reaction in actinians, Rand ('15) says:

> In general an epithelium will not tolerate a free edge. When such an edge arises, accidentally or otherwise, the epithelium extends until, if possible, the free edge meets and unites with some other portion of the same layer or with another epithelium. The essential function of an epithelium is to cover a surface continuously.

The artificial cultivation of epithelial cells (Loeb, '02; Harrison, '10, '14; Holmes, '13, '14; Oppel, '13; Uhlenhuth, '14; Osowski, '14; Hooker, '14 b, and Matsumoto, '18) has shown the migration to be ameboid in character and demonstrated that the cells are stereotropic. The presence of some solid body along which they may migrate is necessary. Loeb ('20 b) interprets the reactions seen in wound healing as essentially reactions to foreign bodies. The stimulus of the foreign body causes surface changes in the cells, leading to ameboid movement. Holmes ('14) had these reactions in mind when he said, page 292, that "cells of the epidermis have an inordinate tendency to lateral spreading." The time necessary to restore the epidermis depends upon the size of the wound. According to Loeb and Strong, a small wound in the skin of the frog will be covered within two or three days. No distinct increase in mitoses is seen until after the second day. Experiments with wounds in tadpoles' skin show conditions similar to those found in the frog. The regeneration of the dermis, on the other hand, is a much slower process. It proceeds first by migration, and later by proliferation of cells, and requires weeks for completion. Even after three months the boundaries of the old wound can be distinguished.

When an autotransplant is fitted into an area denuded of integument, the migration of epidermal cells is reduced to a minimum. In such cases the cells move centripetally from the edges of the wound, and centrifugally from the edges of the transplant, until by fusion they form a connecting layer. The epidermis of the graft and the host becomes continuous within three hours. After several days the line of union is marked by an hypertrophy of epidermal cells, forming a wedge-shaped mass extending down towards the dermis. Figure 11 shows such a formation, although of different origin. By the time the dermal layers of the two regions have united, between three and four weeks after the operation, this formation has disappeared, and the normal thickness of the epidermis is restored. Subsequent behavior of the graft depends upon its kind and its source, and will be described later.

3. *Proliferation of the transplants*

Only those grafts which were taken from the tail showed any growth, i.e., increase in size, of tissue. In some cases the new tissue formed was irregular in shape and bore no resemblance to a tail. In other cases growth resulted in the formation of a miniature tail. In order to avoid any intimation that the growth of tail-skin grafts was an attempt of the tissue to form a new tail or a new body, the word proliferation is used. Proliferation as used here means merely the formation of new tissue by the transplant. The maximum amount of new tissue varied in individual cases from one-tenth of the transplant to more than twice its size (fig. 16). It was not determined what the factors are which inhibit further growth when the maximum is reached. They must be similar to those which halt the growth of all normal tissues and organs. Whatever the cause, the fact that the growth of the transplants is limited is no more remarkable than the limitation of the growth of all normal tissues in any organism.

The rate of proliferation is slightly affected by the amount of illumination. In the transplants kept in darkness proliferation began earlier, proceeded at a greater rate, and came to an end sooner than in the transplants kept in light. Under either condition the final result was the same, due to the longer period of growth in those exposed to light. Another difference was that the new tissue formed in darkness was more irregular or wrinkled than that formed in light, possibly because of the greater rate of growth. These differences were never marked, but the observation of a large number of cases proved their existence.

In a transplant showing the maximum amount of proliferation the original anterior and posterior ends had produced more new tissue than the other two edges, regardless of the orientation of the graft. In twelve cases proliferation was restricted to the originally anterior end, and in twelve others to the originally posterior end. In twenty-two grafts both ends proliferated equally, forming an elongated diamond-shaped mass (fig. 16). A tail-skin graft, therefore, retains its anteroposterior polarization, both ends being able to proliferate new tissue. Lateral prolifera-

tion is slight. The ability of the anterior edge of a piece of tail of an amphibian to proliferate has been reported in the literature several times. Vulpian ('59) observed a very slight proliferation at the anterior ends of isolated tails of frog tadpoles. Later Born ('97), by means of grafting experiments, demonstrated that the anterior edge of tail pieces could produce new tissue. One year later, Harrison ('98) observed proliferation in a tail which had been grafted in reversed orientation. In other words, the anterior end had proliferated tissue. In one case, where the tail of one animal was grafted into the back region of another, the anterior end proliferated more tissue than the posterior end. He interprets this proliferation as an incomplete regeneration of a body, rather than a heteromorphosis.

In addition to polar proliferation, grafts showed also extremely irregular outgrowths. Slender rods, curled or straight, thin sheets growing out at different angles, bulbous and pocket formations are types of such irregular growths. Their distribution over the surface of the graft was general, no area being exempt. Several grafts which possessed outgrowths of all the kinds mentioned presented a grotesque appearance. One of the transplants established union along its right side only, causing it to stand on edge. Proliferation proceeded from both ends which turned toward each other and fused, thus forming an open, hollow cone 4 mm. high. At this point growth ceased, the cone remaining unchanged for months afterward. There was exhibited in such grafts, then, what may be called 'amorphic regeneration' (figs. 5, 7, 14). The size of the graft seemed to have no relation to the rate or the amount of proliferation. Several times small irregular masses of epidermis from the tail, less than 1 mm. across, were transplanted to the back. All of them showed rapid proliferation, until their size had been more than doubled in some cases. It is suggested by this fact that the minimum size of a tail-skin transplant which will proliferate is probably one active epidermal cell.

Histological preparations of transplants which had proliferated new tail tips at both ends showed that the notochord and the nerve cord had been extended in both directions about four-fifths of the length of the new tissue. Both of these structures appeared

like those of a normally regenerated tail (fig. 11). Harrison states definitely that in his grafts the nerve cord was never regenerated, although the notochord was. It may be that the production of nerve cord in the grafts described here was due to conditions of age or environment or both, different from those under which Harrison made his grafts. In several cases proliferation from the originally anterior and posterior ends consisted chiefly of the new notochord with a very thin connective-tissue layer between it and the epidermis. Such outgrowths were cylindrical, from 1 to 6 mm. long, and usually twisted in various ways (fig. 5).

MECHANICAL ADJUSTMENT OF GRAFTS

1. Grafts over eyes kept in the light (series LN), or in darkness (series DN)

a. Tail skin. The first transplantations consisted of tail skin placed over the eyes of animals kept in light. Due to the presence of some muscle tissue and the notochord, and to the presence of the melanophores of the skin, the transplants were usually opaque enough to conceal the eye and, presumably, to inhibit vision. They were grafted in four different orientations, viz., with the originally anterior end either, 1) on the anterior side of the wound; 2) on the ventral side of the wound; 3) on the posterior side of the wound, or 4) on the dorsal side of the wound (table 1). No relation between the orientation of the transplant and its behavior could be determined. The source of the skin was usually the animal's own tail. A few homoiotransplants were made, but they exhibit no differences in the adjustment process. It should be borne in mind throughout the following descriptions that any graft over an eye of a tadpole is necessarily concave on its under side, conforming to the shape of the eye. The history of each transplant may be divided into three periods, according to the changes which are taking place in it.

(1) First or healing period. The first period covers the time of healing and the establishment of union with the host integument, and its duration depends primarily upon the accuracy

TABLE 1

The LN series[1]

NUMBER	SOURCE	ORIENTATION	SYM. OR ASYM.	AMOUNT OF ABSORPTION	PROLIFERATION			
					Ant. only	Post. only	Both ends	Amorphic
10	tail	N	sym	100% Ex.			“	
18	tail	R	sym	100% Ex.				“
27	tail	N	sym	100% Ex.				
31	tail	R	sym	50% Ex.		“		
32	tail	R	sym	33% Ex.				“
43	tail	N	sym		“			“
44	tail	N	sym	100% Ex.				
45	tail	R	sym	sl. abs.			“	
48	tail	N	sym					“
50	tail	R	sym	50% Ex.				“
51	tail	N	sym	50% Ex.			“	
52	tail	N	sym	75% Ex.				“
53	tail	R	sym	25% Ex.				“
54	tail	N	sym					“
57	tail	N	sym	75% Ex.				“
58	tail	R	sym	100% Ex.				
59	tail	N	sym	100% Ex.				
60	tail	N	sym	75% Ex.	“			
61	tail	N	sym					“
62	tail	N	sym	sl. abs.				
63	tail	R	sym					
64	tail	N	sym	100% Ex.				
65	tail	270°	sym					“
66	h. tail	R	sym					“
67	tail	N	sym			“		
69	tail	R	sym					“
70	h. tail	270°	sym	100% Ex.				
71	h. tail	R	sym	100% Ex.				
72	h. tail	N	sym	50% Ex.				
74	tail	R	sym	100% Ex.				“
75	tail	N	sym					“
77	tail	N	sym	sl. abs			“	
78	tail	N	sym				“	
79	tail	N	sym			“		“
80	tail	N	sym	sl. abs.			“	
81	tail	N	sym				“	
82	tail	270°	sym				“	
83	tail	90°	sym	100% Ex.				“
84	tail	270°	sym	100% Ex.		“		
85	tail	N	sym	100% Ex.				
86	h. tail	270°	sym	100% Ex.				“

TABLE 1—*Concluded*

NUMBER	SOURCE	ORIENTATION	SYM. OR ASYM.	AMOUNT OF ABSORPTION	PROLIFERATION			
					Ant. only	Post. only	Both ends	Amorphic
87	h. tail	90°	asym	sl. abs.	"			
88	h. tail	270°	asym	50% Ex.				
89	tail	270°	asym	100% Ex.				
90	tail	270°	asym					"
91	back	N	asym					
92	tail	270°	asym	sl. abs.				"
93	tail	90°	asym			"		
94	tail	90°	asym	sl. abs.				
96	tail	90°	asym					"
97	tail	270°	asym	33% Ex.				"
98	tail	270°	sym			"		
99	back	N	sym					
100	back	N	sym					
101	back	R	sym					
102	back	N	sym					
103	back	R	sym					
104	back	R	sym					
105	tail	270°	sym	100% Ex.		"		
106	tail	270°	sym	100% Ex.				"
107	tail	270°	sym	25% Ex.				"
108	tail	270°	sym	sl. abs.		"		
109	back	N	sym					
110	back	R	sym					

Totals

64	55 tail 9 back			37	3	8	8	24

[1] The abbreviations used in this table are as follows:
Sym., symmetrically placed over the eye
Asym., asymmetrically placed over the eye
% Ex., percentage of exposure of eye after absorption
Sl. abs., slight absorption not reaching eye
Ant. only, proliferation at originally anterior end of graft
Post. only, proliferation at originally posterior end of graft
N., graft placed in original orientation
R., graft placed in reversed orientation
90°, graft placed with originally anterior end ventral
270°, graft placed with originally anterior end dorsal
h., homoiotransplant; all others were autotransplants

of the fitting of the transplant to the wound. If the latter is exactly covered, the healing process, as already stated, is very short, complete union being established by the end of twelve hours. If, on the other hand, the transplant is too small or too large for the wound, the healing process is greatly prolonged and firm union may not be established until the end of thirty-six or forty-eight hours. A graft which is too small has a stretched appearance during this period, as though the epidermal cells, moving out over the wound, exerted a centrifugal pull on the whole mass. This favors firm and complete union of the transplant with the host integument along all its edges, provided the distances between the four sides of the graft and the host integument are nearly equal. If one side is much farther away from the host integument than the opposite one, the former will be prevented from uniting because of being pulled away from the incision (fig. 1). Such a free edge will hasten the absorption process, which will be described in the next paragraph. A transplant of excessive size exhibits a turning under of its projecting edges. If all sides project, an increase in the convexity of the whole graft is produced during the healing period; if only one, the increase is limited to that side. If the overlap is not too great (less than about 2 mm.), the curved-in edges and the edges of the host integument unite. But since such grafts commonly are puckered at one place or another, union is rarely complete. In any event, a transplant too large for the wound is more convex than one of the right size or one somewhat too small and is less likely to be completely united. The first period, then, varies, its length being from twelve to forty-eight hours, at the end of which time the graft is attached to its host along the whole, or nearly all, of its periphery. During this time there is no external sign of vascular congestion, although sections show accumulations of blood cells in the dermis.

(2) Second or adjustment period. Since all living tissues are continually being worn away or used up, the effete elements being replaced through regeneration, it is to be expected that transplanted tissue will undergo similar changes. If the replacement of tissue does not keep pace with wastage, then the transplant

will disappear. But if wastage does not exceed replacement, the transplant will continue to exist. In many of the tail-skin grafts placed over the eye, there was a local disappearance of tissue; that is to say, in a certain area of the graft, the location varying in individual cases, wastage predominated over the formation of new cells. The result was the disappearance of tissue in that area. The word 'absorption,' as used in this paper, is defined as the disappearance of tissue in a small part of the graft, without any intimation as to the method of such disappearance. The word 'adjustment,' because of its broader meaning, has been selected to designate the period in which absorption takes place. During the adjustment period, a majority of the grafts, thirty-seven out of fifty-five, showed absorption in varying amounts—a visible proof that some kind of adjustment in the grafts was occurring. Although the other eighteen grafts did not show this sign, it is believed that they also passed through an adjusting process. The reasons for the non-appearance of absorption in those cases, as will be described later, are probably correlated with a firmer state of union between graft and host, or thicker tissue of the graft.

The beginning of the absorption is seen first in those transplants which have one edge unattached. The free edge shrinks back from the incision, causing the contour of the edge to become convex to the center of the graft (figs. 2, 6, and 18). In symmetrically placed grafts, i.e., where the eye is beneath the center of the graft, the apex of the absorbed area is directed toward the eyeball, no matter which edge was free in the beginning. Absorption continues until the eye is partly or fully exposed to view (figs. 3, 4). The process is then checked, no further absorption taking place. The time at which evidence of absorption appears depends upon the amount of free edge in the beginning. Transplants LN 18 and 75, for example, whose ventral edges were unattached, began to be absorbed during the third day after the operation, and on the tenth day the eyes were entirely exposed. When the length of the free edge is small or when only a corner of the graft is free, the absorption process is delayed. LN 60, with its anterior ventral corner free, illustrates such a condition.

Absorption did not appear in that graft until the end of the fifth day after the operation. On the sixteenth day, about one-half of the eye was uncovered. Absorption then ceased. The grafts which had established complete union all along their edges showed absorption later than any of the others. In LN 44 ten days elapsed before the appearance of absorption. On the twentieth day after the operation the entire eye was exposed (fig. 12). As will be seen from the figure, which is a photograph of the living animal, the graft is roughly crescent shaped. In that condition it remained until death on the one hundred and twenty-seventh day after the operation. The only change that occurred was a slight proliferation of tissue around the edges of the graft. Of the thirty-seven grafts which showed absorption, twenty-six had established complete union. In these twenty-six cases absorption began on the average twelve days after the operation. In the other eleven grafts, each one of which had some free place along its edges, the average number of days preceding absorption was five. Complete union, therefore, delays the absorption process about one week. The delay is usually compensated, however, in the grafts with complete union by a greater rate of absorption when it does begin. Thus in LN 51, with complete union, the first signs of absorption appeared on the fourteenth day. At the end of the eighteenth day about one-half of the eye was exposed, and absorption then stopped (fig. 18). In this case, absorption continued only four days—a period shorter by nearly a week than that seen in other grafts where union was incomplete. There were a few grafts in which absorption began very late in the adjustment period and produced a very small U-shaped area not reaching the eye. It is supposed that in these cases the third period, one of proliferation, began before the absorption had accomplished what it would have, if proliferation had not begun.

Complete union may also cause a second type of adjustment, which was shown by six transplants, LN 64, 74, 83, 86, 105, and 106. In these grafts a circular area near the center gradually became thinner by absorption, and at last was perforated, thus exposing the eye. In none of them were there any signs of in-

fection or other abnormal conditions. The behavior in all of them was similar, absorption appearing in the second week, and continuing, on an average, for seven days. When the process stopped, the opening was about the size of the eyeball (fig. 13). As a rule, then, absorption begins at any free place along an edge of the graft. When there is no free place, it starts at the weakest point on the line of union. When all edges are firmly and completely united, then the center of the graft, which is at the point of greatest convexity and farthest away from the host, is the starting-point of absorption. The place where absorption begins is thus determined by the mechanical state of union between the transplant and the host.

It will be noticed from the foregoing description of the absorption process, first, that the amount was by no means constant, varying from the maximum, which exposed the entire eye, to that which produced only a small U-shaped area at a free place without reaching the eye; and, secondly, that absorption usually began before the close of the second week and came to an end during the third week after the operation. There were only two exceptions to this rule. LN 62 established complete union and thereafter up to the fiftieth day showed no changes. At that time it became loosened near the posterior ventral corner, and absorption proceeded anteriorly along the ventral edge. After a slight withdrawing of that edge, the process stopped and no further changes took place (fig. 6). In the other case, LN 88, with complete union, the middle of the anterior edge broke loose on the thirty-second day. Absorption continued slowly until the fortieth day, when one-half of the eye was exposed. These two grafts were exceptions in another respect, since they never showed any proliferation of tissue. There comes a time, therefore, in the history of the transplant, after which an adjustment by absorption does not occur, and this time, according to averages, is about twenty-one days. It marks the close of the second period.

(3) Third or proliferation period. During the adjustment period, the eighteen grafts which were not absorbed remained without visible changes. Union had been established along all edges. At about the beginning of the fourth week all transplants,

excepting LN 62 and 88, with or without absorption history, began to proliferate. The formation of new tissue by the graft was never observed to occur during the adjustment period. It is assumed that a profound change takes place in the tissues of the graft which makes them incapable of further adjustment and initiates growth. Previous to such a change the connection of the blood vessels of the graft to those of the host has been completed. It is likely, then, that with the normal blood supply restored, the transplant is able to form new tissue. This new activity, recognized by an increase in size of the graft and directly opposed to absorption seen in the second period, predominates during the third period. No further adjustment ever takes place. It is suggested by this fact that the absorption process in a certain part of the graft may be aided by a poorer blood supply in that region than in other regions.

The originally anterior and posterior ends of the graft show new tissue before the sides, and the amount produced at the ends is much greater than that at the sides. In many grafts, however, the proliferation was distributed irregularly over the surface, constituting amorphic regeneration. The limits of the old tissue are easily distinguishable from the new by the larger number of melanophores in the former—a condition which persists for months.

When growth begins it proceeds rapidly for a time, and then almost suddenly ceases. The following abbreviated notes from the records of LN 79 illustrate the history of a typical case of proliferation:

Oct. 4, 1920. Auto-tail-graft over right eye in original orientation.

Oct. 5. Complete union along all edges. During the next two weeks no noticeable changes occurred.

Oct. 20. Outgrowth along posterior edge of the graft.

Oct. 22. Very slight proliferation along the two sides. Noticeable increase in the amount of new tissue at posterior end.

Oct. 24. Posterior outgrowth is distinctly triangular in shape. No increase in lateral growth.

Oct. 30. Posterior outgrowth appears like a normally regenerated tail tip.

Nov. 3. No further increase in proliferation.

Nov. 17. No changes.

Jan. 9, 1921. No changes; animal killed and graft fixed for sectioning.

Proliferation in this case began on the sixteenth day after the operation, continued for ten days, and then ceased. Up to death, over two months after the last recorded change, no further growth took place. In other cases growth continued slowly for a month before stopping.

The transplants which had shown no absorption did not differ from the others in regard to proliferation. Both kinds of grafts passed through the growth period, and at its close reached the final stage of equilibrium. This behavior was constant in all tail-skin grafts, many of which were kept under observation for as long as five months after the last noticeable change had occurred. Morgulis ('09, p. 639) summarized the regeneration of the marine worm, Podarke, as follows:

> There is a lapse of some time, which varies with different individuals, and under different conditions, before new tissue is proliferated; this is followed suddenly by a period of rapid formation of new segments to be in turn followed soon by a period of slower regeneration. Finally the process is brought to a standstill.

The histories of the proliferation of the tail-skin grafts on frog tadpoles and the regeneration of Podarke are thus seen to be similar.

In the DN series (table 2) fourteen transplants of tail skin were made, and the animals kept in darkness. Four of them showed absorption, a lower percentage than that found in the LN series. In two cases the eye was one-half exposed; in the others the absorbed area just reached the eyeball. The adjustment went on in a manner similar to that seen in the light grafts. Therefore, it cannot be said that the absence of light was the direct cause of the smaller percentage of absorption cases. The difference is correlated with the earlier appearance of proliferation. The average number of days at the end of which growth was first observed in the DN series was twelve. In the LN series the average was twenty-one. The adjustment period of the DN grafts was nine days shorter than the same period in the LN grafts. Further, the rate of growth was higher in the DN series, and was indicated by the greater irregularity of the new tissue formed in darkness. It is a well-known fact that in plants the

absence of light results in a higher rate of growth. It is not surprising, then, to find that the same thing is true of animal tissue. Amorphic regeneration is typical of DN grafts. Only three of them showed tail-like regeneration in addition to the amorphic type. In the light series twenty-four out of fifty-five showed the amorphic and nineteen produced miniature tail tips. It seems that the absence of light hastens the adjustment period, allowing

TABLE 2

The DN series

NUMBER	SOURCE	ORIENTATION	SYM. OR ASYM.	AMOUNT OF ABSORPTION	PROLIFERATION			
					Ant. only	Post. only	Both ends	Amorphic
2	tail	R	sym		"			"
3	tail	N	sym					"
4	tail	N	sym					"
5	tail	N	sym	50% Ex.				"
6	tail	N	sym					"
7	tail	R	sym					"
8	tail	N	sym					"
9	tail	R	sym					"
10	tail	R	sym					"
11	tail	N	sym	sl. abs.				"
12	tail	R	sym	50% Ex.				"
13	tail	N	sym				"	"
14	tail	N	sym					"
15	tail	270°	sym	sl. abs.		"		"
16	back	R	sym					
17	back	N	sym					
Totals								
16	14 tail 2 back			4	1	1	1	14

proliferation to get an earlier start, and also increases the rate of proliferation (fig. 14). These two conditions tend to prevent adjustment in those grafts which are slow to show it, and to bring adjustment to a close, in those grafts which have begun to be absorbed, earlier than if they were in the light. With these exceptions, the histories of the LN and DN series were similar.

b. Back skin. The second group of transplantations over the eyes consisted of grafts of integument taken from the back region of the same animal or from another animal. A square of skin from the middle of the back was cut out and grafted over the eye in the same way as with tail-skin grafts. The histories of such grafts differ very markedly from the histories of tail-skin grafts, since the former never show absorption or proliferation. The healing period is essentially like that described for tail-skin grafts, but the subsequent behavior varies according to the source of the tissue. Autotransplants remain unchanged and enter the period of equilibrium after about four days. The line of union is marked by a denser mass of melanophores than elsewhere for several weeks afterward. But in time this condition disappears and the graft becomes indistinguishable from the surrounding skin (fig. 17). Vision is thus permanently inhibited, since back skin is thoroughly opaque. Neither increase in size nor any other indication of growth is shown by such grafts, some of which were under observation for five months. Homoiotransplants of back skin over an eye are not usually successful. When placed flat on other parts of the body, they unite readily, but the healing period is prolonged. This is probably due to the action of the homoiotoxin upon the protoplasm of the graft, which has been shown by Loeb to delay healing of grafts. When the graft is placed over an eye the tissue is forced into a curved position by the convexity of the eye. Because of the delayed healing and the curvature of the new position, homoiotransplants of back skin are not able to establish good union. Usually only one edge succeeds in becoming attached, so that nearly all of the graft soon dies. But the homoiotransplants which did unite completely had histories similar to those of the autotransplants; they showed neither absorption nor proliferation.

The striking difference in behavior between tail-skin and back-skin grafts may be due to the physical difference in structure. Tail skin is mostly epidermis, with a very thin dermal layer, while back skin has a well-developed dermis with many glands and a dense fibrous layer. Therefore, back skin is thicker, more compact and much less plastic than tail skin—facts that may well

explain its indifference to any stimuli tending to change its size and shape.

2. Grafts not over eyes (series LB) or over operated eyes (series LO)

The outcome of the back-skin transplants contributed only negative evidence to the question of a relation between the visual function and the absorption process, because the difference in behavior between the tail-skin and back-skin grafts seemed to be due to the difference in structure. It was then planned to make grafts of tail skin on the back region of tadpoles, not over the eye, to determine whether absorption would ever occur in such cases. These grafts constituted a part of the LB series (table 4). The operation was like that in the other series. The tail skin was fitted to the incision and grafted in place (fig. 15). The orientation was varied and some of the animals were kept in darkness. None of the twenty-two grafts so made were absorbed. They all established complete union along all edges and at the end of two weeks they began to proliferate new tissue. From the anterior end only, growth occurred in three cases; from the posterior end only, in one case, and from both ends equally, in eight cases. Sixteen cases showed amorphic regeneration. Five weeks after the operation, on an average, these LB grafts had reached the period of equilibrium. Thereafter the tissue remained unchanged. There is no doubt that the tail-skin grafts placed on the body region not over an eye differ entirely in their behavior from those placed over an eye. The former never show absorption; a majority of the latter do. When the operation was varied by using back skin, the results were the same as far as the absence of absorption is concerned. Autotransplants and homoiotransplants of tail or back skin on the back region are never absorbed, and only the tail-skin grafts proliferate. These results indicated definitely that the eye in some way is responsible for the absorption of the tail-skin grafts. Whether the relation between the organ and the adjustment of the graft was due to the function or the structure of the eye was still undetermined. It seemed as though the severance of the optic nerve before the graft was placed over the eye would afford evidence on this ques-

tion. Tail-skin grafts were, therefore, placed over eyes whose optic nerves had been cut, and also over empty sockets after the removal of the eyes (the LO series, table 3). In doing the former operation a small piece of the optic nerve, about 1 mm. long was removed, so as to prevent regeneration.

The results of these grafts may be briefly stated. No absorption was seen in any of the LO grafts. The explanation is as follows. After the severance of the optic nerve, the eye rapidly

TABLE 3

The LO series

NUMBER	SOURCE	OPERATION	ABSORPTION	PROLIFERATION			
				Ant. only	Post. only	Both ends	Amorphic
1	tail	op. n. cut	none				"
2	tail	op. n. cut	none				"
3	tail	op. n. cut	none	"			"
4	tail	op. n. cut	none				"
5	tail	op. n. cut	none			"	"
6	tail	eye removed	none				"
7	tail	eye removed	none	"			"
8	tail	eye removed	none			"	
9	tail	eye removed	none	"			
10	tail	eye removed	none				"
11	back	eye removed	none				
12	back	op. n. cut	none				
13	back	op. n. cut	none				
Totals							
13	10 tail 3 back	7 op. n. cut 6 eye removed	none	3	0	2	8

degenerates. At the end of several weeks, postmortem examination showed only the remains of the whitish opaque lens. The operation and the degeneration of the eye which follows greatly lessen and often obliterate the curvature of the conjunctiva, so that grafts placed over such eyes are not under the same conditions as grafts over normal eyes. Those over empty sockets are either flat or slightly concave on their outer surface. The absence of curvature or the destruction of vision in the LO series may have been the reason why absorption did not take place. All

TABLE 4

The LB series

NUMBER	SOURCE	SKIN BENEATH	ABSORPTION	PROLIFERATION			
				Ant. only	Post. only	Both ends	Amorphic
1	tail	“	none				“
2	tail	“	none	“			
3	tail	“	none			“	“
4	tail	“	none				“
6	tail	“	none			“	
7	tail	“	none				“
8	tail	“	none	“			“
9	tail	“	none				“
11	tail	“	none			“	
12	tail	“	none				“
13	tail	“	none	“			“
14	tail	“	none				“
15	tail	“	none				“
16	tail	“	none			“	
17	tail	“	none				“
18	tail	“	none				“
19	tail	“	none			“	“
20	tail	“	none				“
21	tail	“	none			“	“
22	tail	“	none			“	“
23	tail	“	none			“	
24	tail	“	none		“		
25	h. back	“	none				
26	h. back	“	none				
27	h. back	“	none				
28	h. back	“	none				
29	h. back	“	none				
30	h. back	“	none				
31	h. belly	“	none				
32	h. belly	“	none				
34	h. belly	“	none				
35	h. belly	“	none				
36	h. belly	“	none				
37	belly	“	none				
38	belly	“	none				
39	belly	“	none				
40	belly	“	none				
41	belly	“	none				
42	belly	“	none				
43	belly	“	none				

TABLE 4—*Concluded*

NUMBER	SOURCE	SKIN BENEATH	ABSORPTION	PROLIFERATION			
				Ant. only	Post. only	Both ends	Amorphic
44	belly	"	none				
45	belly	"	none				
46	tail	Skin and	none		"		
		glass be-					"
		neath					"
47	tail	"	none				
48	tail..	"	gl. out				
49	tail	"	gl. out	"			
50	tail	"	none		"		
51	tail	"	none				"
52	tail	"	gl. out				
53	tail	"	none	"			
54	tail	"	none				"
55	tail	"	gl. out			"	
56	back	"	none				
57	back	"	none				
58	back	"	none				
59	back	"	none				
60	back	"	none				
61	back	"	none				
62	back	"	none				
63	back	"	none				
		Glass, but no skin beneath					
64	tail	"	none	"			
65	tail	"	none				"
66	tail	"	none			"	
67	tail	"	none				"
68	tail	"	none		"		
69	tail	"	none				"
70	tail	"	none				
71	tail	"	none		"		
72	tail	"	none				"
73	tail	"	none				
			Totals				
71				6	5	10	24

of the grafts established complete union, those of tail skin proliferating in varying amounts, and the others remaining in equilibrium.

Facts in the histories of the transplants in the LN, DN, LB and LO series may be summarized as follows:

1. Sixty-six per cent of all the tail-skin grafts over normal eyes were partially absorbed in one way or another, allowing light to reach the eyes. Complete union of the graft along its edges delayed the absorption, and in 33 per cent probably prevented it. Orientation of the grafts bore no relation to the process, which was followed by a period of proliferation.

2. Autotransplants and homoiotransplants of back skin over the eyes showed neither absorption nor proliferation. Following the healing period, they remained unchanged in size and shape.

3. When the grafts of skin were placed on the back, not over an eye, absorption never occurred. The origin and the kind of skin made no difference in the results. Tail skin alone proliferated.

4. There was no absorption in grafts placed over empty sockets or over eyes whose optic nerves had been cut. Nerveless eyes rapidly degenerate, destroying the curvature of the conjunctiva. Growth of tail-skin grafts proceeded as in the other series.

These facts strongly suggested that the curvature of the eyes over which the grafts are placed is a possible cause of the absorption process. Tail-skin grafts being composed of epidermis with only a very thin dermal layer, are very plastic and respond to the stimulus imposed upon them by the abnormal curved position. If union is not complete or weak at some place, absorption occurs, tending to restore the normal flat condition of the tissue. Some transplants, because of their greater thickness or because of firmer and more complete union than the others, resist the stimulus and are not absorbed. This adjustment of the graft, however, must take place before proliferation begins. At a certain time in its history the transplanted tissue undergoes a profound change, following which absorption is checked and proliferation is initiated. Back-skin grafts, being composed of more compact tissue, with a fibrous dermis, are not affected by the stimulus of abnormal position or are not able to respond to it, and so are never absorbed. This reasoning does not exclude, however, the possibility of a functional cause for the absorption process. It was, therefore,

decided to place skin transplants over artificial eyes on the back of the animal. The mechanical conditions of such grafts would be like those over functional eyes. But the possibility of any influence of the visual function upon the grafts would be completely removed. If the behavior of such grafts should be the same as that of grafts over normal eyes, then the explanation that curvature causes absorption would be substantiated.

3. *Grafts over artificial eyes, series LBE and DBE*

An incision surrounding a square of integument was made in the same way as in series LB. The artificial eyes consisted of hemispheres of glass or celloidin with radii equal to or slightly longer than the radius of the eyeball. The hemispheres were placed on the square of skin with their flat surfaces down, and were covered by the graft of tail or back skin. The orientation was varied and some of the animals were kept in darkness.

As is shown in tables 5 and 6, fourteen out of fifteen tail-skin grafts showed absorption. The amount of illumination made no difference in the result. The average number of days following which absorption began was four. The grafts placed over the largest hemispheres, in which the elevation of the tissue was greatest, began to be absorbed earlier than the others, sometimes during the first and second days. In every case the amount of absorption is recorded as total, because the 'eye' came out as soon as the absorbed area became large enough to permit its passage. Whether the material was glass or celloidin caused no variation in the process. It should be noted that infection or injury of the graft was not the cause of the absorption. All grafts which showed any signs of infection were discarded and their records excluded from the table. After the hemispheres had been freed, the transplants settled down flat and soon afterward began to proliferate new tissue, exhibiting the same characteristics of growth as seen in the previous series. On the other hand, not one of the back-skin grafts was absorbed and none proliferated. All of the nine cases had similar histories, as far as their behavior was concerned. Following the short healing period during which complete union was established, they entered the

TABLE 5

The LBE series

NUMBER	SOURCE	ORIEN-TATION	MATERIAL	AMOUNT OF ABSORPTION	PROLIFERATION			
					Ant. only	Post. only	Both ends	Amorphic
1	tail	R	glass	100% Ex.				“
2	tail	R	glass	100% Ex.				
3	tail	N	glass	100% Ex.				“
4	tail	N	glass	none			“	“
5	tail	R	glass	100% Ex.				“
6	tail	N	cell.	100% Ex.		“		“
7	back	R	cell.	none				
8	back	R	glass	none				
9	back	N	glass	none				
10	back	N	glass	none				
11	back	N	cell.	none				
12	back	R	glass	none				
13	tail	N	glass	100% Ex.			“	“
14	tail	N	cell.	100% Ex.	“			“
15	tail	R	cell.	100% Ex.				
Totals								
15	9 tail 6 back		10 glass 5 cell.	8	1	1	2	7

TABLE 6

The DBE series

NUMBER	SOURCE	ORIEN-TATION	MATERIAL	AMOUNT OF ABSORPTION	PROLIFERATION			
					Ant. only	Post. only	Both ends	Amorphic
1	tail	R	glass	100% Ex.				“
2	tail	R	glass	100% Ex.	“			
3	tail	N	cell.	100% Ex.				
4	tail	N	cell.	100% Ex.				“
5	tail	R	cell.	100% Ex.		“		
6	tail	R	glass	100% Ex.				“
7	back	N	glass	none				
8	back	R	glass	none				
9	back	N	cell.	none				
Totals								
9	6 tail 3 back		5 glass 4 cell.	6	1	1	0	3

period of equilibrium showing no changes during the time of observation, which averaged 120 days.

It might be said that the absorption of the tail-skin grafts over artificial eyes was caused by the presence of foreign material beneath them. To test this possibility, the hemispheres of glass or celloidin were replaced by thin squares of glass cut from the ordinary cover-slip. Otherwise the operations were the same as those in which the hemispheres were used. Even in such grafts slight curvature cannot be avoided. The glass laid on the square of skin inside the incision raises the graft slightly above the surrounding integument. When the edges of the graft begin to unite with the surrounding skin, they are pulled downward. This downward pull on the edges causes a noticeable curvature of the graft. In 40 per cent of these grafts (four out of ten cases) absorption occurred and the glass was liberated. The liberation in two cases was no doubt facilitated by the sharp corners of the glass pressing against the graft. Six grafts showed no absorption, the glass remaining buried. The eight back-skin transplants of this sort were not absorbed, the glass remaining permanently underneath. From these experiments, it was concluded that the presence of foreign material beneath the graft was not the cause of absorption of tail skin, but that the curvature of the graft was the cause. In order to eliminate curvature of the graft entirely, all of the integument within the incision must be removed. When this was done and the thin square of glass placed in the wound was covered by tail skin, no absorption occurred. The grafts do not react to foreign material. Their only activity is proliferation. Flat grafts in any region have never been absorbed. Kendall ('16), reporting on the use of frog skin to cover slowly healing wounds in human subjects, says that the ideal wound to graft is flat. He found that grafts over curved surfaces were usually unsuccessful. In addition to the curvature, the action of the heterotoxin produced in such grafts must also hinder the establishment of union.

The adjustment of a skin graft over an eye is therefore determined by the curvature of that organ. Artificial eyes produce the same behavior as normal functional eyes. The incidence of

absorption cases and the rate of absorption are in direct proportion to the degree of curvature. The mechanical stimulus due to curvature, however, is effective only when the graft is composed of plastic tissue, like tail skin. The compact tissue of back skin is not able to adjust itself. The stimulus is probably a tension of some sort, resulting from the healing process. An appearance of stretching in grafts is very common. The tension acting on the graft leads to a local disappearance of tissue. This absorption in turn relieves the tension, and the graft shows no further adjustment. It may be concluded, then, that the inhibition of the visual function in the frog tadpole by an opaque graft does not effect any regulation of the graft. The adjustment which does occur is purely a mechanical affair. There are two possible reasons why no regulatory effect is seen. First, the eye has lost the power, which it possessed during its organogeny, of causing the overlying skin to become transparent. This loss might be expected, since the eye has completed its development. Secondly, assuming that the eye does act upon the overlying skin, the latter has lost its power of response to such action. This condition of the skin could likewise result from the completion of development. In either case, the high degree of differentiation existing both in the eye and the skin is the fundamental reason why there are no effective interacting forces. The loss of the power of regeneration, of regulation, and of various other processes by an animal at certain periods of its development is a familiar fact. It has been called the law of genetic restriction. On this ground, the absence of any functional regulation in an opaque graft over the eye of a frog tadpole is explained.

LOCAL SPECIFICITY OF INTEGUMENT

1. *Normal integument*

The arrangement of pigment cells in a frog tadpole's tail is quite different from that seen on the back region of the animal. In the skin of the back dermal melanophores and xantholeucophores are very abundant, and, when expanded, they form an intricate network of interlacing processes. These pigment cells

are more or less evenly distributed throughout the skin of the back. Although there are distinct variations in pigmentation between R. catesbeiana and R. clamitans, the foregoing statements hold true for both species. In the skin of the tail the number of dermal pigment cells is very much smaller than in that of the back. Under the binocular microscope individual cells may easily be seen, even during extreme expansion. There may be occasional collections of several melanophores and xantholeucophores within a small area, in which the limits of individual cells are obliterated. But in general the cells are distinct. In R. catesbeiana the masses of melanophores form definite rounded markings and are designated as specific characters for that species. In R. clamitans the spots have very irregular outlines and are not so dense in color and are inconspicuous. The epidermal melanophores of both back and tail are essentially alike, but differ slightly in distribution. On the back they are more numerous than on the tail. As a result of these conditions, the two kinds of skin bear characteristic markings or pigment patterns which easily distinguish them from each other. In the normal tadpole each region continues to produce skin of its own kind. If an area is denuded of integument on the back or the tail, it is quickly covered by new integument of the original type. Where is the mechanism that controls this persistent production of one, and only one, kind of skin? Is it located in the skin itself or in the animal as a whole? Evidence on this question ought to be obtained by transplanting from one region to another and observing what changes, if any, take place. The experiments concerned with the adjustment process afforded an opportunity to study such grafts. After they had been examined, other autotransplants were made. Back skin was grafted on to the tail and belly skin on to the back. The behavior of homoiotransplants from the same regions was also studied.

2. *Autotransplants*

Tail skin grafted on to the back is conspicuous because of the difference between the pigment patterns of the two types of skin. Figures 12, 14, 15, and 17 illustrate such grafts. The visible

changes which take place in tail-skin grafts are of two kinds: 1) those concerned with the adjustment process when the grafts are over eyes, and 2) those concerned with proliferation. None of these changes affect the characteristic appearance of tail skin. The new tissue formed by the grafts is similar in all respects to tail skin. Many of the outgrowths even possessed the shape of a tail and were described as miniature tail tips. The characteristics of the pigment pattern and the translucency of tail skin were preserved in the old tissue and reproduced in the new. One hundred and thirty-six transplants of tail skin on various regions of the back gave uniform results in this respect. Not a single graft failed to preserve its individuality. The periods of observation varied from two to eight months, the average being six. Since the epidermal cells are constantly being cast off and regenerated and since the tail skin is mostly epidermis, it is safe to say that nearly all of the tissue in an old graft, say six months after the operation, has been formed since the graft was placed.

If there is any chemical action of the surrounding skin, or of the tissue beneath, or of the blood and lymph, which tends to change the visible characteristics of the graft, then it is evident that such action is not effective. A more likely assumption is that no such tendency exists, but that the cells of the grafts are self-differentiating, and, if provided with nourishment, reproduce their kind. It is immaterial to such tissue whether the proper nourishment comes through the blood vessels of the back or of the tail. In other words, the mechanism, perhaps the result of a specific chemical group in the protoplasm, resides in the cells themselves, and is not dependent upon any interaction with other mechanisms outside the cells.

Integument from one location on the back grafted into a new position on the back likewise remains unchanged. All of the cases in which back skin was placed over the eye resulted in the graft's becoming indistinguishable from the surrounding skin. Figure 17 shows such a graft three months after the operation. The line of union is marked by a band in which the melanophores are more numerous than elsewhere. This condition disappeared after four months. Grafts placed on other parts of the back

also showed no changes. All of the autotransplants of back skin on the back (thirty cases) gave the same result. No antagonistic interaction between the grafted and the host cells was indicated.

Having proved that tail skin transplanted to the back, and that back skin transplanted to a new position on the back always retain their individuality, logically the next experiment was to make reciprocal grafts; that is, to transplant integument from the back on to the tail. There are some mechanical difficulties in making such grafts. The back skin may be rolled into a cylindrical mass after healing has begun, or may become so wrinkled that union is prevented. However, when the wrinkling is not too great and union is once established, the graft persists. Seven cases were observed. In every one the difference in appearance between the two kinds of skin was very marked, and all of them continued to maintain this difference for months after the operation. The results were so uniform that the making of more grafts of this kind was not deemed necessary. The conclusion is inevitable that back skin, as well as tail skin, will retain its individuality when transplanted into a strange region of the same animal, indicating that there is no effective interaction between host and graft tissue.

The only other region of the tadpole in which the skin is characteristically different from those already considered is the venter. From nearly the whole of that region melanophores are absent, and the number of xantholeucophores greatly exceeds that in any other part of the animal. As a result, the color of the venter is white or cream. When transplanted on to the back, belly-skin grafts present an excellent opportunity for studying any changes in structure or pigmentation that may result. The skin heals readily and firm union is established within twenty-four hours. Such grafts, ten of which were made, are always sharply delimited and never become indistinguishable from the surrounding skin. Because of the transparency of the epidermis, the deeper layers of dermis may be seen with the aid of the microscope, and months after the operation the dermis appears characteristically different from the dermis of back skin. In a very old graft even a casual observer would notice the difference. All

of the ten grafts made were alike in this respect. Therefore, the conclusions drawn from the results of the other kinds of grafts are valid also when skin from the belly is transplanted.

The final series of autotransplants consisted of belly skin grafted on to the tail. There were six of these grafts. It is not necessary to describe their histories in detail, because in general they were similar to those in the other series. The conditions of wrinkling were not quite so pronounced as when back skin was used. The important observation is that all of them preserved their individuality.

It has been shown by reciprocal transplantation that in three regions of the tadpole's body a specificity exists. However such a condition may be explained, whether simply by postulating on good evidence from other sources, a specific protein in the protoplasm, or mystically by assuming the presence of some vital force the fact remains that wherever skin may be successfully transplanted on the same animal, the specificity and not the environment determines its behavior. The specific protein, however, is not different enough from that in another region to set up a violent reaction when the two are brought into contact. If such a reaction did occur, the grafts would be destroyed, owing to the excess of host substance over graft substance.

3. *Homoiotransplants*

Homoiotransplants of integument from the back or belly exhibited striking differences when compared to the same kind of autotransplants. There were two constant features of the eleven homoiotransplants. The first was a vascular congestion, which appeared as a network of blood vessels and spaces, making the graft conspicuously red. Through the microscope the circulation of the blood in this network could be observed. Histological preparations showed accumulations of blood cells massed not only beneath the dermis, but also between the dermis and the epidermis. The time when congestion appeared varied from twenty-four hours after the operation to four days. Its duration averaged three days. Both its appearance and disappearance required twelve hours each. Following the disappearance of

congestion, the graft remained unchanged for two or three days. The second feature of the grafts was a lymphocytic reaction similar to that described by Loeb ('20). It was seen in sections that nearly all of the available spaces between the dermis and the epidermis were filled by lymphocytes. Several weeks after this condition appeared, the fibrous layer of the dermis showed evidence of disintegration and replacement by new tissue. The more less continuous sheet of dermal melanophores found in normal back skin was broken up into isolated irregular pigment masses. The epidermal melanophores had greatly increased in number and melanin granules were frequently abundant throughout the epidermis. Macroscopically, the graft was considerably lighter in color than the surrounding skin, due to the condition of the dermal pigment cells. Sections of grafts from the venter also showed the degeneration of the dermal layer. The lymphocytes were very numerous and the epidermis contained many melanophores, and scattered melanin granules. Dermal melanophores, of course, were absent. The acquisition of black pigment cells by white belly-skin grafts is the subject of the following section of this paper and will be described there.

The congestion and lymphocytic reaction are interpreted as the first steps in a process of replacement of the graft by new tissue formed by the host. The occurrence of such a process is evidence of a rather violent chemical reaction going on between the protoplasms of the graft and host, and a merely specific difference between the two could set up the reaction. This condition confirms the observation of many others that the protoplasm of one individual is chemically different from that of a second individual of the same species. Winkler ('10) made reciprocal homoiotransplants of back and belly skin in Hyla, and found that they retain their individual characteristics. In 1913 Borst presented to the International Medical Congress at London a review of the evidence on the specificity of protoplasm, which had been obtained up to that time. For a list of papers on this subject, the reader is referred to his inclusive bibliography. Later in that year Weigl ('13) reported that homoiotransplants of skin in salamander larvae retain their specific character, as far as color and markings

are concerned. He also found that the transplant metamorphosed in the manner typical of the region of the animal from which it came, without regard to its orientation or to the location of its new position. He concluded that in different regions of the body the skin is self-differentiating, and that the development of a certain color and pigment pattern in a region is not the result of correlation between the whole animal and the skin. The factors determining the typical color and pattern must therefore be formed early in development, and are deposited in the skin. This would explain why skin, transplanted to another region, will develop its own typical markings regardless of the surrounding skin.

In a general discussion of transplantations, Barfurth ('14, p. 579) says:

> Während im Allgemeinen für Transplantationen tierischer und pflanzlicher Objekte die Regel gilt, dass Unterlage und Transplantat ihre Eigenart bewahren, sind aber doch Beeinflussungen der Komponenten aufeinander bei der innigen Vereinigung möglich und unumgänglich.

The striking experiments of Kornfeld ('14) on the transplantation of gills in Salamandra show that there must be a specific substance for gills which causes the grafts to retain their typical form and structure even after undergoing partial absorption and reaching equilibrium.

A very definite statement is made by Thomson ('17, p. 170) to the effect that "there are as many protoplasms as there are individuals, and in homoiotransplants the appearance of a foreign protoplasm in the body calls forth ferments into the circulation which destroy the transplant."

The results which Uhlenhuth ('17) obtained by homoiotransplantation of skin on Amblystoma larvae were like those of Weigl's with Salamandra. Uhlenhuth found that "individual types of yellow spots of the skin of one individual remained unchanged even when the skin was grafted to an individual whose skin developed yellow spots of another type." He concluded that the factor which is responsible for the kind of yellow spots developed is contained in the skin itself and is specific.

Recently L. Loeb ('20, '21) has summarized the investigations that have been carried on by him and his co-workers for the past nineteen years. Having performed several kinds of transplantations on guinea-pigs, rats, rabbits, and fowl, he concludes that all the tissues of an animal contain a specific chemical group, which is responsible for the animal's individuality. He calls this substance the 'individuality differential.' When transplanted to a closely related animal of the same species or to one not closely related or to an animal of another species, this specific substance reacts with the individuality differential of the host. either directly or indirectly, to form a syngenesio-, a homoio-, or a heterotoxin, respectively. The resulting reaction may be studied quantitatively, and is found to increase in violence as the remoteness of the relationship between graft and host increases. This furnishes, therefore, a method of determining the degree of relationship between individuals. In addition to this individuality differential, there are also in the tissues of an animal "contact substances," "which act upon adjoining or distant parts of the body and thus bring about a correlation of functions which makes possible the orderly development and maintenance of the organism" (Loeb '21, p. 163). He concludes that cells and tissues are constantly producing certain substances which play different rôles in the life of the organism as a whole. Some of them, for example, are concerned with preserving the individuality of the whole animal; others act as stimuli upon adjoining tissues and organs; and still others are carried to distant parts of the body before exerting their specific action. The latter are the hormones.

The evidence in favor of specificity of protoplasm due to chemical differences not only between different individuals, but also between different organs and regions of the individual is fairly conclusive. The subject is a part of modern biological thought, and only a few of the researches concerned with it have been mentioned here. However, the possibility that the common tissues, such as muscle, connective, and skin, might contain specific substances in different regions of an individual has received slight attention. Many experiments have shown marked

differences between different regions of an animal's body in their chemical and physiological reactions. For example, when the anterior and posterior ends are differentiated, the animal is said to show 'polarity' or to possess 'axial gradients.' Such terms may be used merely to describe conditions, but when they are employed to express the cause of regional differences, the solution of the problem is only delayed. However, if specific chemical differences in the cells of the regions concerned can be demonstrated, then the explanation of differences in form, structure, and function will be easier.

In the frog tadpole it has here been shown that integument in the tail region is specific and retains its individuality even when transferred to a new soil. Similarly, skin from the back or the belly transplanted to the tail continues to produce back or belly skin, respectively, and preserves its individuality indefinitely. There must be, therefore, in the integument of these regions different substances which are constantly being produced by the cells and which determine the character of the new cells formed.

Two observations in which local specificity of skin in different regions of an animal has been noted have come to the attention of the writer. These are both reported by Schöne ('12). He describes reciprocal autoplastic grafts of belly and back skin in mice, and states that each kind of skin retains its individual characteristics when transplanted to the other region. He then quotes (p. 97) the experience of Marchand. In examining a graft of arm skin on the nose of a man, presumably autoplastic, Marchand found that all the characteristics of arm skin had been preserved two years after the operation. Including the evidence derived from the experiments reported here, there is ground for saying that in frog tadpoles, mice, and human beings a local specificity of integument exists.

ACQUISITION OF MELANOPHORES BY WHITE GRAFTS ON A BLACK REGION

1. Autotransplants

It has already been remarked in this paper that white skin transplanted from the venter to the back acquires black pigment. Although the appearance of melanophores in such grafts varied in autotransplants and homoiotransplants, yet the final result was the same in both cases. An attempt was made to discover how this condition was produced. The experiments of the first group were autotransplants. In these cases the melanophores did not appear until several weeks after the operation. The process was very slow, and in three grafts was not completed at the end of five months. The way in which it began was always the same. Along the line of union between the graft and the surrounding skin, the melanophores began to collect in large numbers, forming a conspicuous black border. Very slowly this border of pigment cells widened, becoming less dense as it covered more and more of the graft. Sometimes the melanophores in the pigmented area were arranged in such a position that their long axes were approximately perpendicular to the nearest edge of the graft. This produced the parallel arrangement illustrated by figure 22, although this condition was never as prominent in autotransplants as it was in homoiotransplants. Another arrangement was found in some grafts, in which the melanophores were more numerous along the four diagonals of the graft than between them. Differences in size, as well as in the distribution, of the cells were also evident. The largest melanophores were at the line of union, and the smallest ones were found at the other limit of the pigmented border. In this region also large and small masses of melanin granules were abundant. Some of the largest masses were about the size of melanophores, but without the definitive shape of such pigment cells. Occasionally, however, a large melanophore of typical appearance could be seen among these masses. Centripetal spreading continued until the whole area was pigmented, giving to the graft a gray color. As time went on, the shade of gray deepened, due to an increase in the number

of melanophores, although the graft never, even in the oldest cases, became as dark as back skin. Hasty examination indicated that nearly all of the melanophores were epidermal, and a study of sections confirmed that observation. Only a few scattered dermal melanophores were found in old grafts. But epidermal pigment cells were abundant. Many of the epidermal cells contained pigment granules in varying amounts. Some of them were nearly filled by pigment, and could easily be interpreted as 'young' melanophores. Another proof that the pigmentation of such grafts is chiefly epidermal may be obtained by peeling off the epidermis. Such removal, causing the disappearance of pigmentation, is always followed by a regeneration of epidermis containing melanophores. Belly skin grafted on to the tail becomes pigmented in the same way. The parallel arrangement of the melanophores, the concentration of the pigment cells along the diagonals, and especially the presence of melanin granules throughout the epidermis were features that were observed.

2. *Homoiotransplants*

Homoiotransplants of white skin on the back likewise became pigmented, but the process was different in several respects. From the six cases examined, the records of the following three are of special interest:

Graft LB 32 was taken from the belly of a large tadpole of R. clamitans (80 mm. long) and placed on the back of a small animal (52 mm. long) of the same species. Twenty-four hours after the operation the graft was pigmented. The melanophores were not numerous, and were evenly distributed. Therefore the graft was only a trifle darker than when it was placed. The parallel arrangement near the edges was very prominent. Melanin granules and irregular masses of pigment were not to be seen. At the end of twenty-eight hours the graft was fixed for sectioning. The preparations showed the presence of melanophores in the slightly thickened epidermis. These were indications that the epidermis had migrated from the surrounding skin carrying with it melanophores.

Grafts LB 35 and 36 were reciprocal: that is, belly skin from no. 35 was grafted to the back of no. 36, and belly skin from no. 36 was grafted to the back of no. 35. The animals were of the same appearance, species, and size (45 mm. long). The histories of the pigmentation process in these two grafts were alike. The vascular reaction was

very weak, of short duration, about thirty-six hours. From the end of that time to the fortieth day after the operation no changes were noticeable. On the fortieth day, a concentration of melanophores along the edges of both grafts was recorded. Following that appearance the pigmented border gradually widened, spreading over the grafts, until, on the eightieth day, the last clear areas were pigmented, and the whole of each graft was dark gray. During this period, pigment granules were abundant in the epidermis; many irregular masses of melanin could be found, and parallel arrangement of the melanophores was not at all prominent. Sections of LB 36 fixed on the ninetieth day showed that the dermis was being replaced by the host; that a few dermal melanophores were present, and that the epidermal melanophores and melanin granules were abundant.

When contrasted with the first of the three cases described, these latter two grafts show striking differences. In the first place, there was a difference of seventy-nine days in the length of the pigmentation process. At the end of the longer period, the number of melanophores in the graft was very large, causing a deep shade of gray, while after the shorter period, the melanophores were few, although evenly distributed. Secondly, melanin granules and irregular masses of pigment in LB 35 and 36 were very abundant, while in the other case they were negligible. Thirdly, the sizes and the ages of the animals were very different. The first graft was taken from a two-year-old-tadpole and placed on an animal of half that age. Therefore, the animals were not closely related. The other grafts were interchanged between animals of the same age, size, and appearance, belonging to a group of tadpoles which had been collected from the same source. These are good reasons for believing that they were closely related.

The other homoiotransplants may be arranged between these two extreme cases. The duration of the process was about six weeks. The melanophores were often arranged parallel to each other, and pigment masses and granules were usually not present, especially in the early history of the grafts. The concentration of melanophores along diagonal lines was very frequent. Material sectioned at different stages of pigmentation always showed the presence of epidermal melanophores. Only a few dermal melanophores were found. In the older grafts the replacement

of the dermis was observed. But in none of the sections was there conclusive evidence as to the origin of the melanophores. Before proceeding to a discussion of these results and a possible explanation of them, a brief review of some work done on other animals will be given.

Carnot et Deflandre ('96) grafted small pieces of black skin into unpigmented regions of guinea-pigs, and observed the spreading of pigment from such grafts into the surrounding white skin. The process was slow, requiring weeks before the pigment became noticeable. They further found that the rate of extension of the pigment varied according to whether the host was an albino or a dark colored animal. In the latter case, the rate was described as 'rapid,' while in the former it was extremely slow. This result indicated to them that the extension of pigment was not dependent upon the grafted cells alone, but upon the available supply of melaniferous substances in the host as well. Their account implies that pigment is formed in situ as the result of some stimulus from the graft's acting upon the neighboring skin.

In the following year Loeb ('97) reported that when pieces of black skin from guinea-pigs' ears are grafted to a white region denuded of skin, the pigment spreads out from the graft into the white skin; also that when the reciprocal graft was made, the white skin became pigmented, the process beginning at the edges of the graft and proceeding to the center. From these and later experiments (Loeb and Strong, '04), he concludes that the epidermal melanophores arise from ordinary epidermal cells which have become filled by pigment and become branched, and that they are also capable of rapid migration. Winkler ('10) found that the white skin of frogs or tadpoles transplanted to a black region became black. He believed that the pigment is formed in situ, and that the formation of pigment is an inherent function of the epidermis.

In order to discover any variations in the process that might be found in autotransplants and homoiotransplants, the experiments of Loeb were repeated by Sale and Seelig. They both used guinea-pigs. Sale ('13) found that an autotransplant of black skin on a white region extends its pigment into the sur-

rounding skin, but that a 'homoeotransplant' of the same kind loses its pigment. Seelig ('13) performed the reverse operation and reported that both autotransplants and 'homoeotransplants' of white skin on a black region became pigmented. No explanation of the process is given. Recently Dawson ('20) made grafts of this sort on Necturus. Pieces of pigmented skin from the tail and of white skin from the venter were interchanged. Although it is not definitely stated, it is believed that all of his grafts were autoplastic. He found that the white grafts became partly pigmented. The center of each graft remained clear. The single graft of black skin on the venter began to lose its pigment ten weeks after the operation. At the end of his observations a large irregular central area was free from melanophores. This is contrary to Sale's grafts, which preserved their pigment and extended it into the surrounding skin. Only in homoiotransplants was the pigment lost.

All of these experiments agree in showing that unpigmented skin grafted into a pigmented region of the same animal becomes pigmented. Dawson is the only one to observe that the pigmentation is chiefly epidermal, as it is in frog tadpoles. The two explanations that have been offered to account for the process are, first, that the epidermal melanophores have migrated (either actively, or included in the migrating epidermis as a whole) from the host skin into the graft. Active migration of the melanophores independently of other epidermal cells has not been observed in my transplants, and that method will not be considered here. Secondly, the melanophores have arisen in situ from epidermal cells. Observations of the surface of homoiotransplants in frog tadpoles leads to the belief that the melanophores are carried along by the migrating epidermis, over the graft. The parallel arrangement of the melanophores and the gradual centripetal spreading seem to support this belief. The possible methods of such epidermal migration are four: 1) the graft may be 'overgrown' by a thin epidermal layer; 2) the epidermis of the graft may be 'undergrown' by the invading layer of epidermal cells; 3) the epidermis of the graft may be gradually replaced by an advancing layer of host epidermis; 4) the migrating host

epidermis may intermingle with the graft epidermis. In each method the melanophores would be carried along by the migrating host cells. From the surface appearance and from the fact that the new pigmented layer may be peeled off, it seemed likely that the overgrowth was the method realized in the case of some grafts. However, an examination of sections, both of early and late stages of the process, failed to give conclusive proof. In the case of the twenty-four-hour process, there were indications in some sections of two distinct layers of epidermis, only the outer containing melanophores. In other sections, no such condition could be found, although the epidermis was slightly thicker than normally. In still other cases the epidermis of the graft over a region about 2 mm. from the line of union was considerably thicker than it was in the central region, indicating that the advancing epidermis had intermingled with the graft epidermis. No evidence in favor of the second and third possibilities was found. The only safe conclusion that could be drawn from the sectioned material was that migration from the host epidermis into that of the graft had taken place. Which of the four methods prevailed remains undetermined, although overgrowth seems most likely.

Evidence of the opposite kind came from the grafts LB 35, LB 36, and others like them. Their history did not favor the migration theory at all. The presence of so many pigment granules and irregular pigment masses pointed to a formation in situ. There was no indication of overgrowth or intermingling in any of the sections. It will be remembered that these animals (LB 35 and 36) were undoubtedly close relatives. Therefore, the difference in their specific protoplasms was not very great, and the reaction caused by the grafting was weak. Equilibrium was quickly established, and the slow appearance of pigmentation was probably the result of formation in situ. Assuming that all epidermal cells are potential melanophores and that the surrounding skin exerts a stimulus upon the graft, such formation may be explained. In the twenty-four-hour process (LB 32) relationship between the host and the graft was remote, causing a violent reaction. Actual union was established only by the

epidermis, which showed signs of having overgrown the graft. In other words, the homoiotransplant acted as a foreign substance placed in a wound, over which the epidermis migrated, as it would over any wound. The melanophores were carried along by the migration.

The facts that pigment is constantly being formed in normal epidermis and that epidermal cells migrate rapidly over a wound are well known. Evidence of the specificity of protoplasm in different individuals and the reactions set up between them has been demonstrated by Loeb. Such reactions tend to delay healing, and in proportion to their violence. Because of this delay, the epidermal cells surrounding a homoiotransplant will migrate over it, thereby closing the wound in the shortest possible time. Melanophores are carried along and the graft is pigmented. When the graft animal is closely related to the host, almost immediate healing takes place, the graft being accepted by the host, so that migration of epidermis is not necessary. Melanophores are then formed in situ as the result of the normal growth activity of the integument. In brief, both methods of pigmentation probably exist. Which one predominates, depends on the kind of transplant made. In autotransplants formation in situ is chiefly responsible for pigmentation, but in homoiotransplants and heterotransplants epidermal migration is the primary cause. Both methods concern epidermal melanophores, but only the former method can give rise to dermal pigment cells. When applied to the observations of Loeb, Winkler, Sale, Seelig, and Dawson, this hypothesis seems to hold. It also explains the behavior of the grafts on frog tadpoles. Further experiments will determine its validity.

PIGMENTATION OF THE CONJUNCTIVA CAUSED BY INJURY

In examining a group of tadpoles obtained from a dealer, one animal was found with several scars on its head. The left eye was gone, the wound having been covered by a thin regenerated layer, and the right eye was partly concealed by pigmentation of the conjunctiva. Attention is called to the fact that in frog larvae the external structure of the eye is very different from that

of the adult. The outer portion of the wall of the eyeball, the cornea, is not attached to the overlying integument. The latter fits over the eye smoothly, is transparent, and is homologous to the corneal conjunctiva of the adult eye. Figures 19 and 20 of De Waele's paper ('01) illustrate this condition, which is found also in fishes. The conjunctiva consists of an epidermis two or three cells thick and a very thin dermal layer.

A study of the abnormal conjunctiva revealed the presence in it of xantholeucophores, as well as dermal and epidermal melanophores. The xantholeucophores were fewest and the epidermal melanophores were most numerous. The only noticeable feature of their arrangement was the general and even distribution. The animal was isolated and kept under observation for four months, during which time the pigmentation did not change. It was evident that the abnormality had been caused by an injury to the conjunctiva. If this was true, it should be possible to duplicate the condition in the laboratory. Five healthy normal animals were selected. Their eyes were carefully examined and were found to show no defects or pathological conditions. No pigment cells of any kind were present in the conjunctivas. By means of a fine-pointed needle the conjunctiva of the right eye of each animal was scratched in many places on the surface, and in two cases was punctured. Twenty-four hours afterward epidermal melanophores were distributed over the conjunctiva in three animals, including both punctured cases, and showed distinct radial arrangement. This arrangement continued into the surrounding skin about 2 mm. back from the edge of the conjunctiva. The other two eyes showed epidermal melanophores in the outer half of the conjunctiva only, the central region being clear. Radial arrangement was marked. At the close of the second day, they were pigmented over their whole surface, so that all five cases were similar in appearance. By the end of four weeks the number of epidermal melanophores had increased only slightly and dermal melanophores were appearing around the edges of the conjunctiva. All indications of radial arrangement had disappeared. Xantholeucophores first appeared during the eighth week after the operation. By this time

the dermal melanophores were present throughout the conjunctiva, although by no means so numerous as epidermal melanophores. Subsequent changes were very slow and involved only a gradual increase in the number of pigment cells. A count of the pigment cells in the conjunctiva on the tenth week gave the following totals: ninety-two epidermal melanophores, twenty-one dermal melanophores, and sixteen xantholeucophores. The left eyes of these animals remained normal.

The experiments were repeated on five other animals with the same results. It was found, further, that there was a minimum degree of injury, following which pigmentation was produced, and that pigmentation was probably restricted to that region of the conjunctiva which had been injured. The latter observation was furnished by a single case, in which only the ventral half became pigmented. It was assumed, therefore, that only the ventral half had been sufficiently injured. The determination of relations between the stimuli and the reaction will be the subject of further investigation.

The experimental production of pigmentation in the conjunctiva, such as has been here described, has an important bearing on several subjects. In the first place, it furnishes further evidence on the mechanism of epidermal wound healing. There is no doubt that the epidermal melanophores which were present in the conjunctiva twenty-four hours after the injury were carried along by the migrating epidermal cells from the surrounding skin. The centripetal movement of epidermis occurred in response to the stimulus produced by the wound. This reaction has been discussed elsewhere. The radial arrangement of the cells and their sudden appearance can be explained in no other way. The subsequent increase in the number of epidermal melanophores, as well as the appearance of the dermal melanophores and xantholeucophores was probably the result of formation in situ. This secondary step, one of mitotic multiplication of integumentary cells, is like that which takes place in normal regeneration of skin. The writer believes that this is more probable than that the increase of pigment cells is due to continued migration.

In the second place, the persistence of melanophores in a region which is normally transparent and free from pigment cells shows that a profound change has occurred in the integument, or in the eye, since the time of early development. It was pointed out earlier in this paper that at a certain stage in the development of the eye the overlying ectoderm is thinned, loses its pigment, and becomes transparent. If the ectoderm is removed, the regenerated layer will become transparent, or if it is replaced by ectoderm from another region, transparency will be produced. Lewis ('05) speaks of this process as 'corneal clearing.' However, if the eye is removed entirely previous to this time, no such reaction of the ectoderm occurs. A definite influence of the eye structure upon the ectoderm is thus demonstrated. Since at a much later time pigment is tolerated in this region, proof is obtained that either the eye has lost its power to exert its stimulus upon the overlying skin or else the skin is unable to respond to such a stimulus—a conclusion reached from the study of the skin grafts over the eye. Another set of grafts showed that in the different regions of the body the integument is specific, having lost its embryonic totipotence. The conditions found in the conjunctiva support these conclusions. The tissue of the conjunctiva is specific also, but when it is badly injured, the repair of the wound must be made by the surrounding skin. The cells of that tissue are specific, and when they form a new covering for the eye, they will retain their specificity. Pigment cells which have been carried along will persist, and new ones will be formed as in the normal growth activity of the integument. There are better reasons to believe, therefore, that the skin has lost its power to respond to the stimulus arising from the eye, although the possibility that the eye exerts no stimulus upon the skin is not excluded.

Finally, the pigmentation of the conjunctiva resulting from an injury recalls the appearance of pigment in regions of hypertrophy. There are several kinds of cancers in which the production of pigment is very marked. Some of them are called melanotic because of excessive pigment production. In a study of the origin of cancers, it has been shown that chronic inflammation

may result in abnormal pigmentation. By the rubbing or injection of various substances, such as paraffin oil, xylol, agar-agar, olive oil, and sudan III, into the skin of guinea-pigs, rabbits, and rats, a hypertrophy of the skin was produced, accompanied by an increase in the pigment content (Brosch, '00; Ribbert, '04; Fischer, '06; McConnell, '07). Schultz ('12) described a case of excessive pigment formation of inflammatory origin in human skin. He observed that the chromatophores, as well as other kinds of cells, were stimulated to proliferate. It seems from these results that whenever skin cells are stimulated to multiply at a rate considerably above normal, pigment formation is increased at a still greater rate. In spite of many attempts to explain such reactions, an account satisfying all the known conditions remains to be given. The possibility that sarcomatous pigmentation is due to stimuli fundamentally similar to the appearance of pigment cells following an injury to the conjunctiva is merely suggested here without drawing any conclusions. More evidence on the whole subject of hypertrophy of integument and chromatophores is necessary.

GENERAL SUMMARY AND CONCLUSIONS

A. Mechanical adjustment of grafts over eyes

1. Over the eyes of tadpoles of Rana catesbeiana and R. clamitans varying in length from 20 to 100 mm., opaque skin was transplanted in a manner calculated to demonstrate the existence of any regulatory interaction between graft and eye tending to restore the function of the eye.

2. In 66 per cent of the operations in which tail skin was placed over the eye, the grafts were absorbed in a way which tended to expose the eyes. Back-skin grafts over the eyes were never so absorbed.

3. Tail skin and back skin placed on other parts of the body were never absorbed.

4. Tail-skin grafts over hemispheres of glass or celloidin ('artificial eyes') were absorbed in the same way as grafts over normal functional eyes. Back-skin grafts so placed were not absorbed.

5. Tail-skin and back-skin grafts over thin glass plates were not regularly absorbed. The foreign material is not the stimulus for absorption.

6. The absorption of tail-skin grafts over normal eyes, as well as over the convex 'artificial eyes,' is caused by the curvature of the eyeball or of the artificial eye, respectively. The more compact structure of back skin prevents the absorption of back-skin grafts. The curvature of the graft during the healing period causes a tension, which is the mechanical stimulus for absorption.

7. In direct contrast to back-skin grafts, all tail-skin grafts proliferated new tissue. In some cases miniature tail tips were formed, while in others amorphic regeneration predominated. The anterior ends of such grafts, as well as the posterior ends, are able to regenerate a tail tip containing notochord and nerve cord.

8. Following the proliferation period, all grafts enter a state of equilibrium in which there is no increase in their size.

9. There is, therefore, no functional regulation of skin grafts over eyes. This lack of correlative regulation is the result of the high state of differentiation and specificity attained by the skin and the eye.

B. Local specificity of integument

1. Integument from the tail, back or belly transplanted to another region of the same animal preserves its individual characteristics indefinitely.

2. Homoiotransplants of integument preserve their individuality only temporarily, their tissues ultimately being replaced by regenerated tissue of the host.

3. The integument of frog tadpoles is therefore locally specific, and is self-differentiating when transplanted to new soil on the same animal.

C. Acquisition of melanophores by white grafts on a black region

1. Autotransplants and homoiotransplants of white belly skin on the back or tail region acquire melanophores.

2. In autotransplants the acquisition is chiefly the result of formation of pigment from epithelial cells in situ.

3. In homoiotransplants the pigmentation is chiefly the result of epidermal migration which carries along the melanophores from the surrounding skin. It is followed later by formation of pigment in situ, as in the normal growth activity of integument.

D. Pigmentation of the conjunctiva caused by injury

1. When the conjunctiva of frog tadpoles is extensively injured by scratching or pricking, the regenerated tissue is pigmented, first by epidermal melanophores and later by dermal melanophores and xantholeucophores.

2. The persistence of such pigmentation in the conjunctiva is further proof that no correlation between the eye and the overlying integument exists in tadpoles 20 mm. or more in length.

3. The epidermal melanophores are carried along by the migrating epidermis. The second step in the pigmentation process is probably a mitotic multiplication of integumentary cells, including the formation of pigment cells, and is the result of the normal growth activity of the integument.

E. Reactions of the melanophores

1. Expansion of the dermal melanophores is caused by a low temperature (near 0°C.) independently of illumination, by darkness, by a 0.1 per cent chloretone solution, by anoxemia, and by the low metabolic rate of the whole animal coincident with a moribund state.

2. Contraction of the dermal melanophores is caused by a high temperature (near 35°C.) independently of illumination, by light, and by a return of normal environmental conditions after the removal of the effects of chloretone and anoxemia. Contraction is also coincident with the recovery from a moribund state.

3. Either expansion or contraction usually requires about thirty-six hours for completion, although the reactions begin within twenty minutes after the initiation of the stimulus. Oxygen deficiency brings about complete expansion in about one-half hour.

4. It is therefore probable that any stimulus which lowers the metabolic rate of the whole animal also causes expansion of the melanophores, and any stimulus causing an increase in the metabolic rate causes a contraction of the melanophores.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 to 4 Drawings of graft LN 18 at successive periods in the adjustment process. *s*, scar in host integument where incision was made. The upper edge of each figure represents the anterior end of the graft. × 7.

1 Graft LN 18 as it appeared on the second day after the operation.

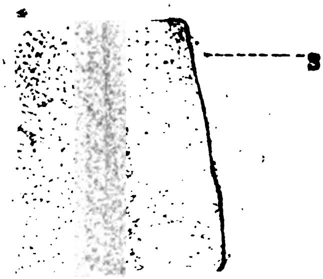
2 Same graft one week after operation.

3 Same graft two weeks after operation.

4 Same graft six weeks after operation, showing the maximum amount of proliferated tissue that was formed.

5 Graft LO 5 four months after the operation. Proliferation from both anterior and posterior ends is shown, as well as amorphic regeneration over the surface of the graft. The anterior outgrowth is cylindrical and twisted back on itself, while the posterior one is tail-like. No absorption occurred. × 7.

6 Graft LN 62 four and one-half months after the operation. The ventral edge is unattached and has shrunken back toward the eye—a condition which persisted. × 6.



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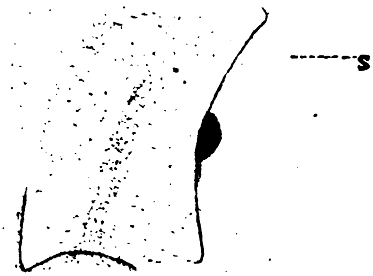

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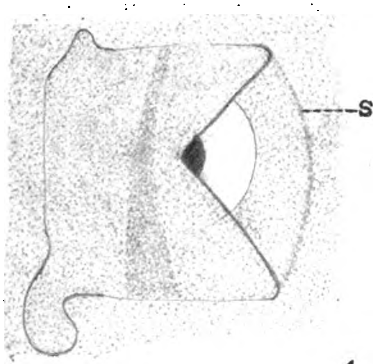

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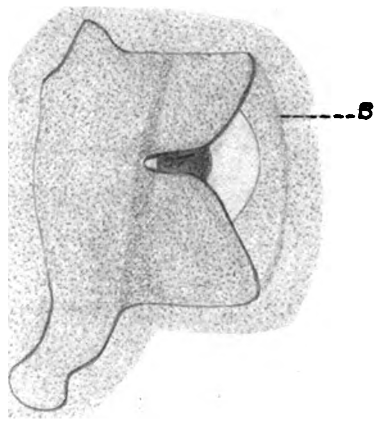

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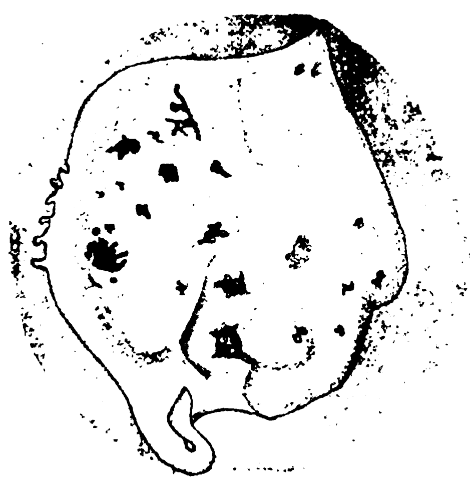

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PLATE 2

EXPLANATION OF FIGURES

7 A transverse section of graft LN 79, illustrating amorphic regeneration. Only grafted tissue is shown. The notochord (*n*) is in two parts, the larger being the old notochord and the smaller being the regenerated notochord. The nerve cord is seen just beneath the notochord. Due to the irregularity of the proliferated tissue, there are several spaces lined by epidermis, all of which open to the outside, however. × 34.

8 Transverse section of graft LB 10 three days after the operation. Beneath the graft is seen the integument of the host (*hi*) with many blood cells in the dermis. Blood cells have also penetrated the notochord (*n*) of the graft. Subsequent behavior of such autotransplants results in the lining of the space between graft and host by epidermis, with an opening to the outside somewhere. × 51½.

9 Transverse section of graft LO 2 five weeks after the operation. It will be noticed that the dorsal side of the graft (lower edge in figure) has become continuous with the skin of the host, due to establishment of union distally. The other side has united proximally. × 38.

10 Transverse section of graft LO 2 near the posterior edge, showing the point of union on the dorsal side. The epidermis on the under side of the graft and the epidermis of the host, covered by the graft, have become continuous, thus lining the cavity. × 45.

11 Tangential section, nearly longitudinal, of graft LN 78, showing boundary between old grafted tissue and the new proliferated tissue. *n*, notochord of old tissue; *rn*, regenerated notochord; *nc*, regenerated nerve cord. × 97½.

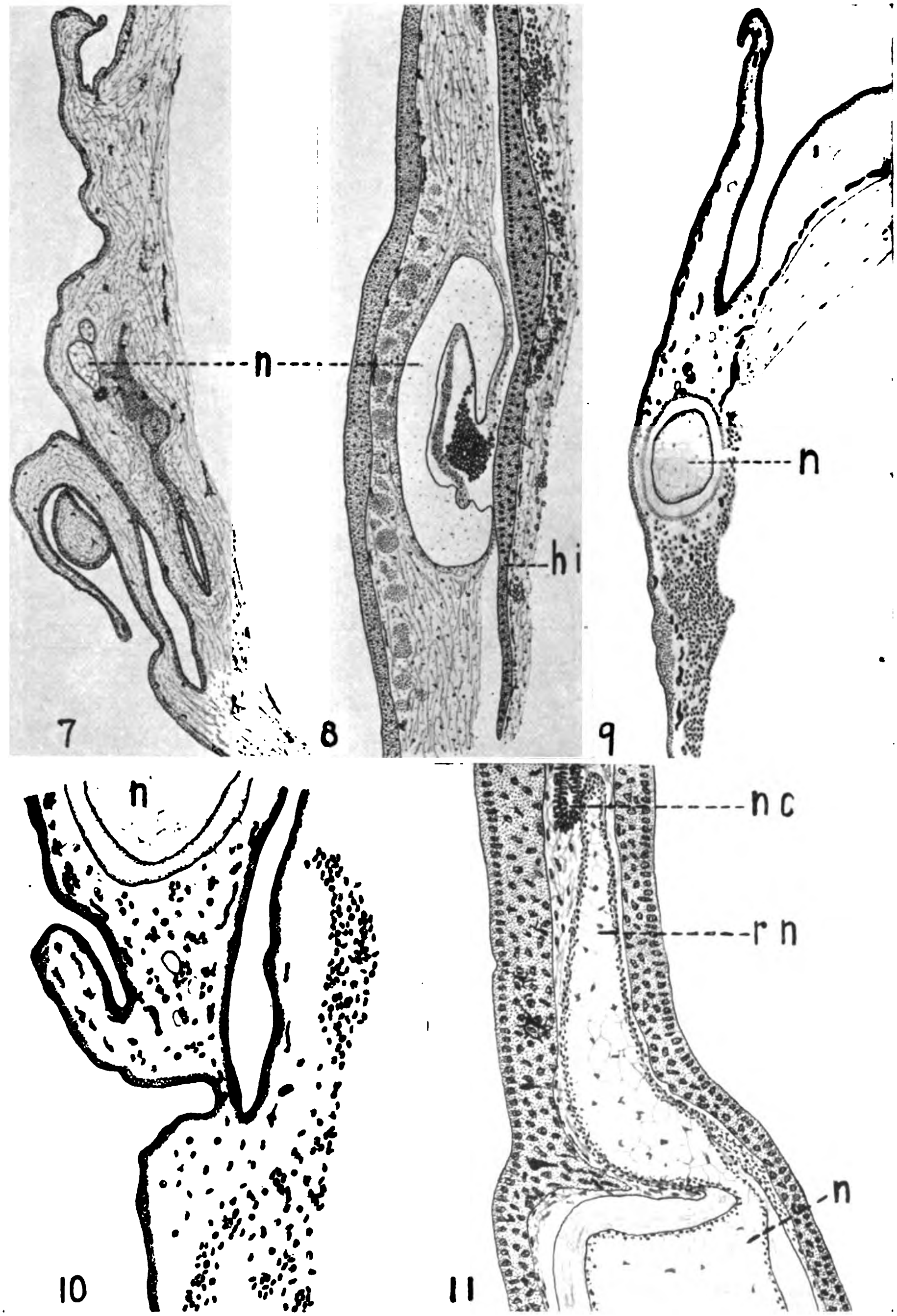
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PLATE 3

EXPLANATION OF FIGURES

12 Photograph of graft LN 44, taken twenty-six days after the operation. The only change up to death, on the 127th day, was a slight increase of proliferated tissue at the ends. × 3.

13 Photograph of graft LN 64, taken eleven days after the operation. The darker region surrounding the central opening is due to an abundance of melanophores there. × 3.

14 Photograph of graft DN 2, taken fourteen days after the operation. Proliferation has begun and is easily seen at the corners of the graft. The irregularity of the proliferated tissue is noticeable. × 3.

15 Photograph of graft LB 9, taken four months after the operation. Slight amorphic regeneration is shown. No absorption occurred and the characteristic features of tail skin were preserved. × 3.

16 Photograph of graft LN 78, taken three months after the operation. Proliferation from both ends, absence of absorption, and preservation of tail-skin characteristics are the features of this graft. Refer to figure 11. × 3.

17 Photograph of graft LN 104 taken three months after the operation. The back-skin graft over the eye has become almost indistinguishable from the surrounding skin, but the tail-skin graft has proliferated and preserved its characteristics. × 3.

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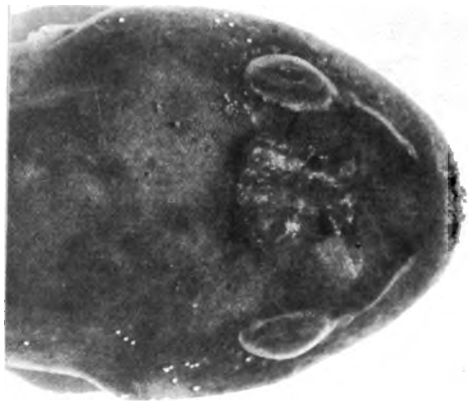

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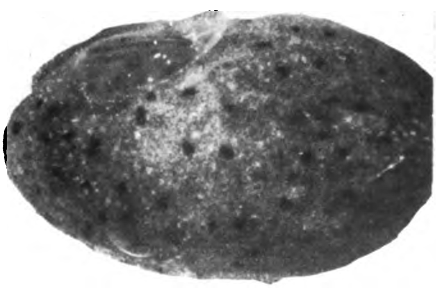

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PLATE 4

EXPLANATION OF FIGURES

18 Photograph of LN 51 taken sixteen days after the operation. Absorption began along the ventral edge. Two days after this photograph was made, about one-half of the eye was exposed in a dorsal view. No further absorption occurred. × 3.

19 Photomicrograph of dermal melanophores of an animal which had been kept in ice-water (0°C.) for four days. An intricate network of melanophore processes entirely obliterates the limits of individual cells. × 293.

20 Photomicrograph of dermal melanophores of an animal which had been kept in ice-water (0°C.) for two hours. The cells, which at the beginning of the experiment were completely contracted (fig. 21), are shown to be about one-third expanded. × 293.

21 Photomicrograph of dermal melanophores of an animal which had been kept in warm water (35°C.) for two days. The cells are maximally contracted. × 293.

22 Photomicrograph of epidermal melanophores of graft LB 34, showing the parallel arrangement of the melanophores and masses of melanin granules. × 320.

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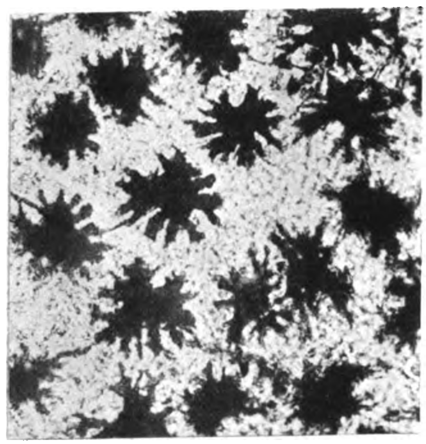

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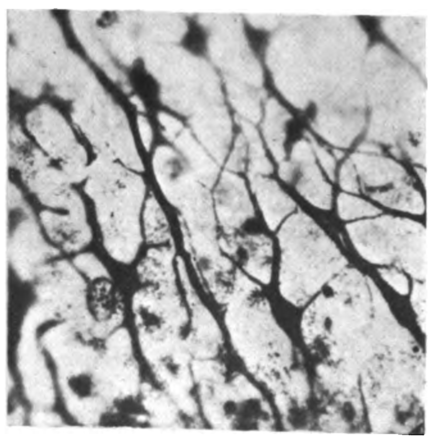

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Resumen por el autor, Leon S. Stone.

Experimentos sobre el desarrollo de los ganglios craneales y el sistema de órganos sensoriales de la línea lateral en Amblystoma punctatum.

Los experimentos llevados a cabo por el autor han consistido en la extirpación de las placodas y la cresta neural. La cresta neural se origina en el tubo neural, en el márgen dorsal de la fusión de los pliegues neurales; en el desarrollo normal, todas estas células, con la excepción de una pequeña parte de ellas, se transforman en el "mesectodermo" emigrante que se extiende ventralmente sobre el mesodermo de los arcos viscerales, envolviéndolos, y finalmente se sitúa en sus superficies medias, en las cuales forma los cartílagos del esqueleto visceral.

La extirpación de las células de la cresta produce ausencia o defectos en la mandíbula, cuadrado, porciones anteriores de las trabéculas, y en todos los cartílagos branquiales, con la excepción del segundo basibranquial. Este último se origina a expensas del mesodermo situado a lo largo de la pared anterior de la cámara pericárdica. La ausencia de la cresta neural en la región branquial está acompañada siempre de pequeñas branquias externas, que contienen una porción subnormal de tejido conectivo. La extirpación de las placodas epibranquiales de los nervios VII, IX y X produce ausencia de los ganglios y nervios viscerales especiales. El sistema cutáneo general deriva en gran parte, si no enteramente, de las placodas, mientras que el sistema visceral general deriva aparentemente de la cresta neural. Cuando se extirpan regiones del ectodermo anteriores y posteriores a la placoda auditiva en el momento de cerrarse los pliegues neurales, la línea del cuerpo, los primordios occipital y supraorbital y sus ganglios de la línea lateral correspondientes faltan. Todos los grupos de los órganos de la linea lateral parecen originarse a expensas de primordios separados.

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EXPERIMENTS ON THE DEVELOPMENT OF THE CRANIAL GANGLIA AND THE LATERAL LINE SENSE ORGANS IN AMBLYSTOMA PUNCTATUM[1]

L. S. STONE

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NINETY FIGURES

CONTENTS

INTRODUCTION

The experiments set forth in this paper were undertaken for the purpose of extending our knowledge concerning the part which placodes and neural crest play in the formation of cranial ganglia and nerves, and also to determine, if possible, the extent of their

[1] Read before the American Association of Anatomists, March 25, 1921.

contribution to the formation of the mesoderm and the exact origin and fate of the 'mesectoderm' tissue. Experiments have never before been applied to the solution of these problems, all of our knowledge having been obtained from studies made upon successive developmental stages of normal embryos.

In the present study of the early stages of development it has been found that cells are given off for the formation of certain ganglia by epibranchial, lateral-line, gasserian, and ophthalmic placodes. From the early appearance of the neural crest in the dorsal portion of the neural canal at the time of the closure of the neural folds the crest cells have been followed as they migrate over the mesoderm of the visceral arches, increase in number and finally arrange themselves upon the median and ventral sides of this mesoderm, where they form the cartilages of the visceral skeleton except the second basibranchial. The distinctness of the early crest-cell groups and placodes makes it possible to remove these structures by employing the operative methods now so commonly used by investigators upon amphibian embryos. It is also possible to remove areas of ectoderm from early embryos in which the placodes have not yet become prominent enough to be identified, and thus to ascertain how early these placodes in the ectoderm are laid down as definite entities.

Much confusion arose in the earlier descriptions of placodes by the failure to distinguish clearly between ectodermal thickenings in relation to the gill clefts and those related to the formation of the lateral-line system. Not until von Kuppfer ('91) clearly pointed out the distinction between dorsolateral and ventrolateral, or epibranchial placodes, was there hope of obtaining light on the formation of the components of cranial ganglia. Descriptions of cells breaking off en masse from the epibranchial placodes and being added to portions of the cerebral ganglia have repeatedly appeared in the anatomical studies of all types of vertebrates, especially among the fishes and amphibians (van Wijhe, '82; Beard, '85; Froriep, '85; Platt, '96, and Landacre, '10 and '12). Landacre has assigned a definite function to these epibranchial placodes. He has shown in Ameiurus ('10) that the special visceral portion of the IX ganglion appears to come from

the epibranchial placode of the first true gill. Since Herrick ('07)[2] had found only gustatory fibers arising from the visceral ganglion of IX, Landacre naturally concludes that the epibranchial placode of IX gives rise to the ganglionic cells from which the gustatory fibers arise. He further expresses the opinion that this is the function of the epibranchial placodes in all types of vertebrates (Landacre, '12), since these placodes occur only on those nerves of VII, IX, and X which contain special visceral fibers, and since the relative size and growth of the epibranchial placodes in Lepidosteus and Ameiurus seem to be in relation to the area and time of appearance of taste buds supplied by fibers from special visceral ganglia. Coghill ('16) finds conditions in Amblystoma which seem to indicate that the visceral ganglia of VII, IX, and X all receive masses of cells from the epibranchial placodes.

There is also evidence that there are other placodes besides those concerned in the formation of lateral-line ganglia, i.e., general cutaneous placodes. Platt ('96) notes in Necturus that an ectodermal thickening above the eye appears to be concerned in some manner with the formation of the ophthalmicus profundus V nerve. A similar fact has been recorded by Coghill ('16) in Amblystoma and by Landacre ('12) in Lepidosteus, where in early stages of the formation of the ganglion of the ophthalmicus profundus V there is a distinct anchorage to the ectoderm over the eye. The former made the interesting observation that it was during this period, in which the anchorage is intact, that root fibers make their connection with the brain. However, neither of the latter investigators satisfied himself as to the significance of this early contact. Coghill ('16) also found in the earliest of his stages described, the 'non-motile,' an area of adhesion between the distal end of the gasserian ganglion and the skin. It is ventral to the primordium of the preauditory lateral-line organs and is not so extensive nor so intimate as the adhesion of the ophthalmic ganglion and skin.

[2] A note included in Landacre's paper ('07) by C. J. Herrick on the distribution of the IX nerve of Ameiurus melas. The conclusions from this reference are found in Landacre's paper ('12), page 3.

In connection with the general cutaneous component of X, Coghill ('16) finds in the 'non-motile' stage an intimate relation between an ectodermal thickening and a loose aggregation of cells upon the lateral aspect of the lateral-line ganglion of X. This cluster of cells, which he identifies as representing the jugular ganglion, is slightly more condensed in its most rostral portion where an incipient root is forming, which does not, however, at this stage reach the brain.

Lateral-line primordia. Platt ('96), Landacre ('10), Coghill ('16), Goette ('14), and others have shown that a different system of placodes is concerned in the formation of the lateral-line ganglia of VII, IX, and X. Not all of the investigators agree as to the exclusive derivation of these ganglia from placodes, for some have expressed the belief that neural crest also enters into their formation.

The primordia of the lateral-line system have been shown very clearly by Platt ('96), Landacre ('10), and Landacre and Conger ('13) to have origins independent of the auditory vesicle. However, anterior and posterior prolongations of the auditory thickening have been observed in many forms, and to these Landacre ('10) applies the term of 'preauditory and postauditory' placodes. He does not have a clear idea as to the relation of these placodes to the sensory lines, for in the case of the preauditory placode (Landacre and Conger, '13) it seemed to disappear by a process of degeneration before the appearance of the lateral-line primordia, and in the case of the postauditory placode ('10) it loses the characteristic cell arrangement and does not give rise to the lateral-line organs. Moreover, in Lepidosteus Landacre and Conger ('13) fail to find any postauditory placode.

Although separate origins have been given to each of the groups of the lateral-line sense organs by various investigators, none have described such an early pattern of the lateral-line system as the one observed in Necturus. According to Platt ('96, p. 491), the plan of this system is early laid down in three longitudinal lines on each side of the embryo, connected by intersegmental cross-lines with special differentiations at points of intersection, and out of this pattern certain portions are retained in the

final system. However, when one compares this description with skin amounts of early stages of Amblystoma, another interpretation seems to be more plausible. No such pattern can be found in Amblystoma embryos, but whenever the contour of underlying structures makes its appearance on the surface certain transitory thickenings appear due to mechanical molding, as one finds between somites, gill swellings, around the early optic vesicles and the early limb bud. It seems possible, therefore, that most of the early pattern described in Necturus has no significance in the formation of the lateral-line system.

Neural crest. It is generally accepted that portions of the cranial ganglia are derived from the neural crest; in fact, early investigators of cranial nerve problems made the crest cells their sole source of origin. Landacre ('10) would derive all general cutaneous ganglia and the general visceral system exclusively from neural crest.

In view of the fact that there have appeared descriptions of extensive wanderings of neural crest, it seems strange that so little emphasis has been put upon such an outstanding feature. The literature contains comparatively few descriptions of any complete investigations of the growth of the neural crest outside of its supposed connection with the formation of cranial ganglia. In most of the descriptions of the early stages of developing cranial ganglia the assumption that the crest cells were concerned only with the formation of ganglia and nerves has been so general that many investigators have been led to overlook the fact that any further wandering of these elements occurs. However, various careers have been assigned to them by a few investigators.

Marshall ('78), working on the chick, and van Wijhe ('82), on the selachians, were among the first to observe that the anterior part of the neural crest, which could not be identified with the formation of ganglia, gradually disappeared. They could not determine its final fate. Kastschenko ('88) went a step farther in suggesting that the cells which were lost from the neural crest did not degenerate, but added themselves to mesenchyme. Additional descriptions of the neural crest then followed, by Goronowitsch ('93) in birds and Platt ('96, '97) in Necturus. Both

agreed that a greater part of the neural crest, augmented at the margin of the gill clefts by a proliferation from the lateral ectoderm, becomes a wandering mass of ectodermal cells to which Platt gave the name 'mesectoderm' and that this wandering mass of ectodermal cells is the origin of certain mesenchymal tissue. It was Platt, however, who suggested that the branchial cartilages, surrounding connective tissue, and the anterior portions of the trabeculae were derived from the 'mesectoderm.' In respect to the branchial cartilages, this agrees with von Kuppfer ('95) on Petromyzon, although he claims that the wandering ectodermal cells are derived from deeper layers of ectoderm in situ. Furthermore, Dohrn ('02), working on Torpedo, and Brauer ('04), on the Gymnophiona, agree in the main with Platt's description of the mode of formation of branchial cartilages, but they ascribe the sole origin of the cartilages to the neural crest.

Recently Goette ('14) has discussed the formation and development of the 'ectomesoderm,' as he calls it, in Siredon (Amblystoma tigrinum) which also illustrates what he finds in Petromyzon and Torpedo. He confines the anlagen of the 'ectomesoderm' to the ectoderm in the regions of V, VII, IX, and X, which he claims forms visceral skeleton, the outer gill muscles, and the surrounding connective tissue. To the neural crest, epibranchial placodes, and lateral-line placodes belongs the function of the formation of the cranial ganglia and nerves.

From this summary of the literature it is evident that there is much disagreement concerning the formation of cranial ganglia and the origin and further distribution of the wandering masses of cells of ectodermal origin.

In the light of these facts, an investigation of this problem was suggested by Prof. R. G. Harrison on an animal, such as Amblystoma punctatum, which would lend itself to an experimental analysis of all the factors involved. It gives me pleasure to express here my appreciation to Doctor Harrison for his kind criticisms and suggestions during its progress.

Since the completion of the present investigation, there has appeared a paper by Landacre ('21) on the fate of the neural crest in Plethodon glutinosus, a urodele. In so far as the visceral

cartilages are concerned, the morphological findings in this paper correspond very closely to the observations recorded in the present study of Amblystoma. In Plethodon the manner of the migration and disposition of the neural crest upon the branchial arches is similar to that described by Platt ('97). In addition to contributing to the ganglionic portions of V, VII, IX, and X, the neural crest is described as forming the greater portion of the mesenchyme in the ventral part of the head and in the branchial region, the anterior portions of the trabeculae, Meckel's cartilage, the palatoquadrate bar, and all the branchial cartilages except the second basibranchial.

MATERIAL, METHODS, AND NORMAL DEVELOPMENT

All the experiments have been made upon embryos of Amblystoma punctatum in stages ranging from 21 to 27 as shown in figures 1 to 4. The technique employed in the operation is similar to that already described by Harrison ('18). The special types of operation employed in this investigation will be subsequently described under separate sections.

In order that the experiments may be more clearly interpreted, a description of the stages in the normal development of the neural crest and of certain placodes will first be given. This description was obtained chiefly from a series of dissections supported in the more minute details by serial sections. In order to accomplish the dissections, embryos which had been preserved in the ordinary corrosive sublimate acetic mixture were placed in a 5 per cent aqueous solution of nitric acid, where they were kept from twelve to twenty-four hours for the purpose of softening the brittle ectoderm and at the same time rendering it pliable. They were then placed under the binocular microscope, and by means of a small pair of operating scissors and suitable needles a continuous incision was made up the middorsal line and down the midventral line. The two halves of ectoderm were carefully removed, stained lightly with haematoxylin, cleared in the usual manner, and mounted in damar on glass slides. After such treatment the positions of ectodermal thickenings can be observed accurately.

The dissected embryos were in some cases preserved in glycerin after they had been stained with haematoxylin. However, the neural crest may be more satisfactorily observed unstained in water or alcohol, because its grayish-brown color sufficiently differentiates it from the surrounding tissue up to a certain stage as will be described later.

Although the picture of the crest cells and placodes is approximately constant for each stage, occasionally normal embryos whose external appearances indicate similar age disclose upon dissection noticeable differences in the early rate of growth of the crest cells or placodes. This difference may even be confined to the same individual where, for example, the advance of the trunk lateral-line primordia of the one side lags conspicuously behind the primordia of the other side for a distance of one or two somites.

Stage 21

Neural crest. A layer of neural crest, light brown in color, over the dorsal and dorsolateral portions of the medullary tube extends from a point above the eye to a point above the posterior border of the second somite (fig. 1) where it is continuous with the crest cells in the spinal region. Ventrally there are two distinct proliferations separated by a constriction lying approximately midway between the two ends. The more anterior proliferation becomes the band which later wraps around the mesoderm of the hyoid arch.

Where the dorsal margin of the mesoderm of the branchial region meets the lateral surface of the medullary tube there is a trough-like depression, filled in by a deep longitudinal ridge of ectoderm extending from above the primitive hyomandibular cleft to near the first somite, where it becomes continuous with less marked ridges over the somites. This is represented by dotted lines in the figure.

Figure 13 is a frontal section passing through the longitudinal ectodermal ridge and the anterior and posterior extremities of the layer of neural crest. The considerable thickness of the ectodermal ridge can be seen on either side in the region of the

hind-brain. A cap of crest cells, whose base extends as a wedge between the fused neural folds, lies in the region of the mid-brain. These cells are represented by well-formed nuclei surrounded by a pigmented cytoplasm in which lie fine, closely packed yolk granules which are similar in size to those of the neural canal. The mass of cells lies close to the ectoderm and extends laterally a short distance over the walls of the mid-brain. Over the region of the hind-brain a similar small cap can be seen lying in the same relation to the ectoderm and brain, except that its base does not appear in this plane of section to be wedged between the fused folds.

Placodes. Figure 1 shows an area over the dorsal border of the eye, which represents the position of a very slight thickening of the ectoderm where cells are given off later to the ophthalmic ganglion. It may be said here that throughout the different stages studied, this ectodermal thickening never acquires a thickness comparable to that of other placodes. Many times in the dissections the area was thinner than the surrounding ectoderm because of the fact that its cells adhered to the loose ganglionic mass of cells forming the ophthalmic ganglion. This thickening may be called the ophthalmic placode.

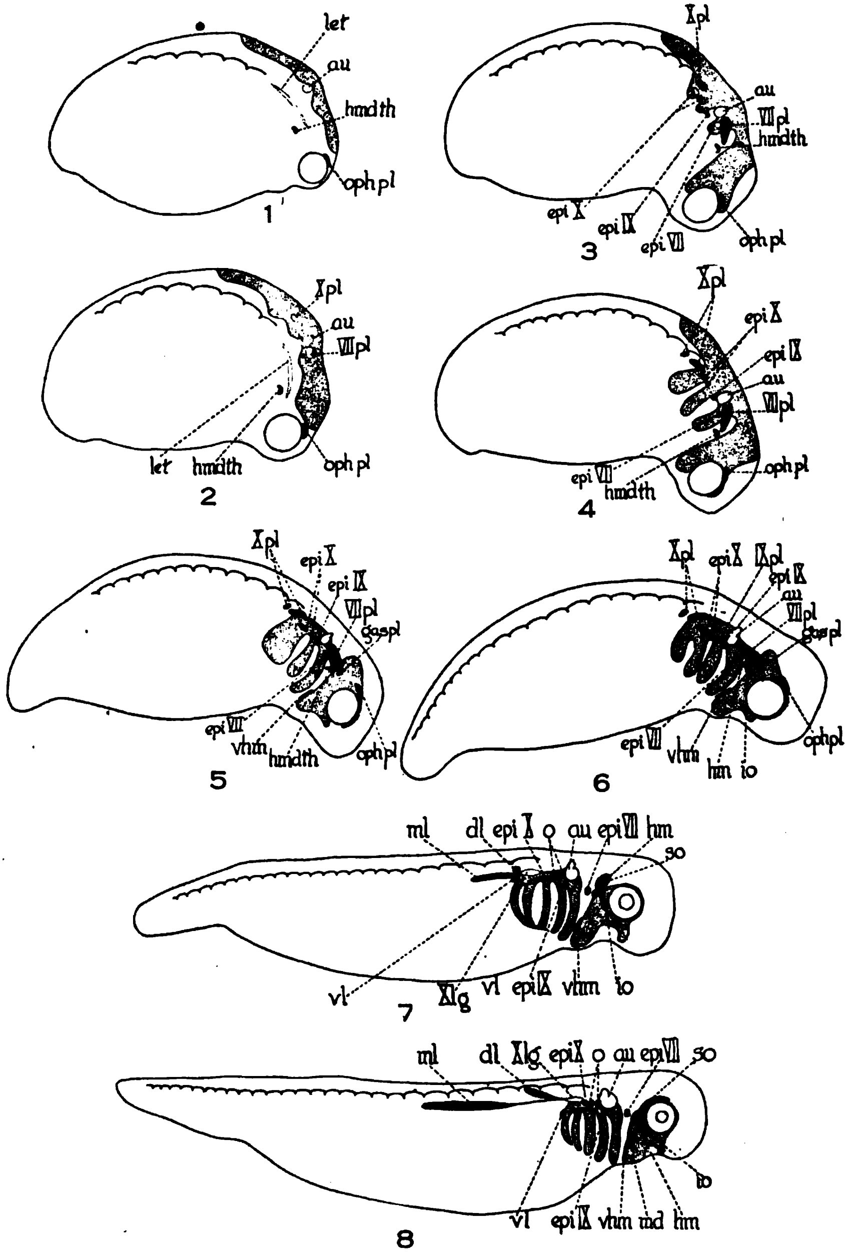
Below the anterior extremity of the longitudinal ridge lies a small ectodermal thickening in the dorsal extremity of the hyomandibular cleft, which can be detected at this stage. This small ectodermal thickening takes on the contour of the cleft at this point, giving it approximately a crescent shape. In the position of the anterior portion of this thickening there later arises the primordium of the infra-orbital group of lateral-line sense organs, while in the position of the posterior portion there later arises the hyomandibular group.

Stage 23

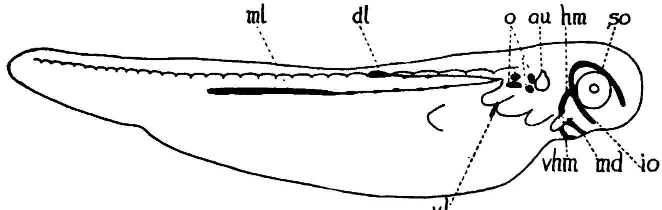
Neural crest. This stage shows a decided ventral growth of the neural crest over the lateral walls of the brain (fig. 2). Above and posterior to the dorsal border of the eye an extensive proliferation has taken place, carrying the neural crest downward toward the primitive mandibular arch. Figure 14 shows above

ABBREVIATIONS

alv., alveolaris VII nerve
an., angular group of sense organs
au., ear
buc., ramus buccalis VII
br., brain
bv., blood vessel
c. c., neural-crest cells
ch., notochord
chy., ceratohyal
dl., dorsal body line of sense organs
e., eye
ect., ectoderm
epi. VII., epibranchial placode of VII
epi. IX., epibranchial placode of IX
epi. X., epibranchial placode of X
ex. g., external gill
gas. g., gasserian ganglion
gas. pl., gasserian placode
jg., jugularis VII nerve
hh., hyohyal
hm., hyomandibular group of sense organs
hmd. th., ectodermal thickening in dorsal hyomandibular cleft
hy. a., mesoderm of hyoid arch
hy. cc., hyoid crest-cell group
hy. m., hyoid muscles
io., infra-orbital group of sense organs
l. e. t., longitudinal ectodermal thickening
mb., mandible
md., mandibular group of sense organs
md. a., mesoderm of the mandibular arch
md. cc., mandibular group of crest cells
md. m., mandibular muscle
md. V., truncus mandibularis V
ment., mentalis VII nerve
m. l., midbody line of sense organs
ms., mesoderm
na., nose
o., occipital group of sense organs
op. v., optic vesicle
oph. g., ophthalmic ganglion
oph. pl., ophthalmic placode
oph. p. V., ophthalmicus profundus V nerve
ph., pharynx
pl., palatinus VII nerve
q., quadrate
s., somite
so., supra-orbital group of sense organs
sup. VII, ramus ophthalmicus superficialis VII
th. m., thoracicohyoideus muscle
tmp. m., temporalis muscle
tr., trabecula
v. hm., ventral hyomandibular group of sense organs
vl., ventral body line of sense organs
1–2 bb., first and second basibranchial
1 bb. cc., first basibranchial crest-cell group
1–4 br. a., mesoderm of first to fourth branchial arches
1–4 br. cc., first to fourth branchial crest-cell groups
1–2 cbr., first and second ceratobranchial
1–4 ebr., first to fourth epibranchial
1 s., first somite
1 int. r., first intersomitic ridge
VII g., facial ganglion
VII l. g., facial lateral-line ganglion
VII pl., facial lateral-line placode
IX lg., glossopharyngeus lateral-line ganglion
IX pl., glossopharyngeus lateral-line placode
IX vis. g., glossopharyngeus visceral ganglion
X lg., vagus lateral-line ganglion
X pl., vagus lateral-line placode
X vis. g., vagus visceral ganglion
X vis. t., vagus visceral trunk



Figs. 1 to 8 Camera-lucida drawings of stages 21, 23, 25, 26–27, 30, 33, 35, and 35+ of embryos of Amblystoma punctatum, showing development of neural crest, ectodermal placodes, and primordia of lateral-line sense organs. Neural crest is stippled and placodes and primordia are solid black. × 10. For abbreviations see page 430.



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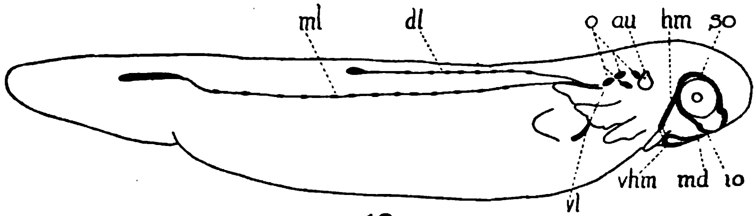

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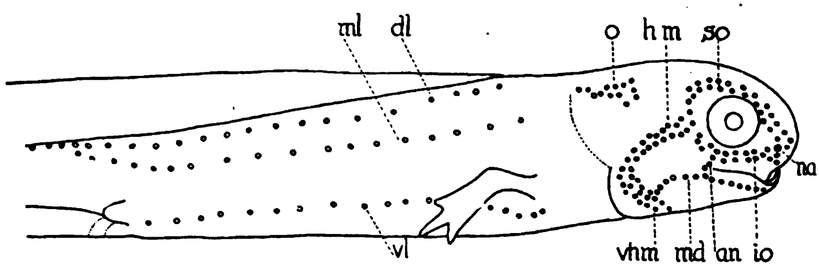

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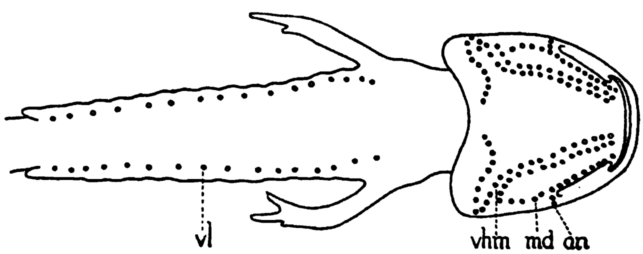

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Figs. 9 and 10 Camera drawings of stages 36 and 37–38, showing further development of lateral-line primordia. × 10.

Figs. 11 and 12 Camera drawings of lateral and ventral views, respectively, of stage 46+, showing final distribution of lateral-line groups of sense organs. The gills have been removed. × 10. For abbreviations see page 430.

the longitudinal ectodermal ridge small portions of the neural crest descending upon the lateral walls of the hind-brain connected dorsally by a cap of cells still wedged tightly between the ectoderm and brain.

Posterior to the mandibular arch are the two prolongations of the neural crest which appeared in stage 21. Approaching the

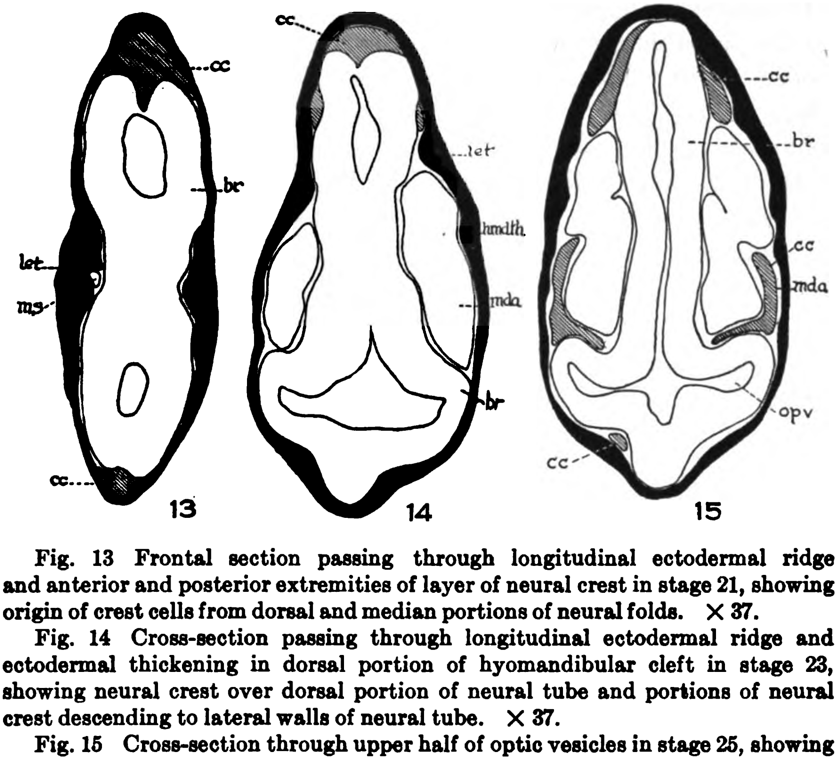


Fig. 13 Frontal section passing through longitudinal ectodermal ridge and anterior and posterior extremities of layer of neural crest in stage 21, showing origin of crest cells from dorsal and median portions of neural folds. × 37.

Fig. 14 Cross-section passing through longitudinal ectodermal ridge and ectodermal thickening in dorsal portion of hyomandibular cleft in stage 23, showing neural crest over dorsal portion of neural tube and portions of neural crest descending to lateral walls of neural tube. × 37.

Fig. 15 Cross-section through upper half of optic vesicles in stage 25, showing crest cells descending along sides of neural tube and over mesoderm of mandibular arches and over anterior portion of right optic vesicle. × 37.

first somite is an additional proliferation which later covers the mesoderm of the second, third, and fourth branchial arches. In this stage the neural crest has extended posteriorly (fig. 2) to a position above the anterior half of the fourth somite.

Placodes. The ophthalmic placode lies in relatively the same position as in stage 21. Its indefinite borders outline a slightly

thickened portion of the ectoderm which is in close contact with the crest cells at its posterior border. The longitudinal ectodermal ridge (fig. 2) is still prominent, but extends slightly more anteriorly and less posteriorly than in the previous stage. The thickening in the dorsal portion of the hyomandibular cleft is slightly larger, but still remains crescentic in shape (fig. 14). The approximate positions of two placodes, anterior and posterior to the probable position of the auditory placode, are indicated by dotted lines. That which lies just anterior to the auditory placode gives rise to part of the lateral-line ganglion of VII. That which lies posterior to the auditory placode gives rise to part of the lateral-line ganglion of X. The positions of other placodes cannot be exactly located in sections of this stage by the appearance of any ectodermal thickenings.

Stage 25

Neural crest. A further very rapid ventral growth of the neural crest has taken place, the most pronounced proliferation involving those cells which are in the region over the dorsal border of the eye (fig. 3). The upper portion of the mandibular arch as far as the middle of the posterior border of the eye is covered by the neural crest. A growth of the neural crest passes over the dorsal border of the eye. A section passing through the upper half of the optic vesicles (fig. 15) shows on one side along the anterior border of the latter a transection of the ventral proliferation of the crest cells lying close to the ectoderm. Posterior to the optic vesicles on either side may be seen the descending crest cells loosely packed together, wrapping around the anterior and lateral surfaces of the mesoderm of the mandibular arch. Over the dorsal border of the hind-brain region lies a thin layer of crest cells which is continued upon the lateral walls as a more compact band of cells. This group of crest cells descends along the anterior border of the auditory placode to the mesoderm of the hyoid arch. A distinct though more slender band of crest cells along the posterior border of the auditory placode approaches the mesoderm of the first branchial arch, extending as far ventrally as the anterior band. Still further

posteriorly, a somewhat broader proliferation, covering the anterior border of the first somite, descends ventrally on the gill mesoderm to the level of the crest cells on the first branchial arch. The neural crest continues posteriorly above the dorsal border of the somites along the dorsolateral border of the neural canal to a position in the middorsal line above the middle of the third somite.

Placodes. The longitudinal ectodermal ridge has disappeared at this stage, due to the rapidly descending neural crest and the growth of the visceral arches. The extent of the area of the ophthalmic placode is about the same as in stage 23. The thickening in the dorsal portion of the hyomandibular cleft has changed but little in size. A placode approximately triangular in shape, situated anterior to the auditory placode, can be seen rapidly approaching the hyomandibular cleft from above. This is the placode which gives rise to a large part of the lateral-line ganglion of VII by contributing large numbers of placodal cells from its posterior extremity (fig. 16). The epibranchial placode of VII, judging from its position in subsequent stages, is probably situated just below the posterior border of this placode. This probable position is circumscribed by dotted line in figure 3. On the posterodorsal border of the hyomandibular cleft is a slight thickening of the ectoderm, which is identified as the epibranchial placode of IX. However, no placodal cells are splitting off from the ectodermal thickening to form a ganglion (fig. 16). In the dorsal portion of the first branchial cleft there is a small thickening of the ectoderm, which is possibly the position of an epibranchial placode, which gives rise to part of the sensory visceral ganglion of X, although no cells are splitting off at this stage. The possible position of the other epibranchial placode of X is also circumscribed by dotted line in the figure. Posterior to the auditory placode and anterior to the first somite, a slight thickening in the ectoderm is identified as the placode which gives rise to most of the vagus lateral-line ganglion.

Stage 26–27

Neural crest. In the description of the following stages, the descending bands of neural crest will be designated by the visceral arches upon which they lie.

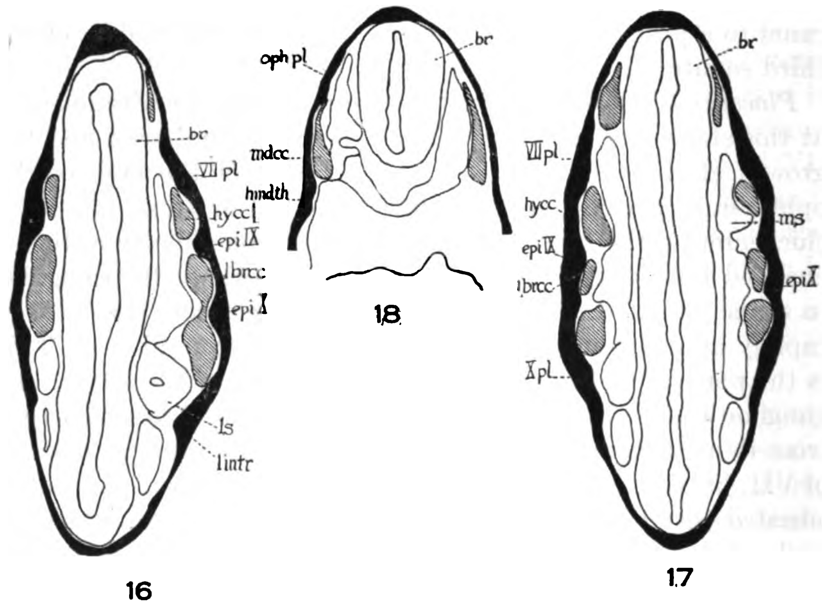


Fig. 16 Frontal section, showing in stage 25 crest cells over dorsal portion of right optic vesicle and over mesoderm of hyoid and branchial arches. Also shows positions of VII lateral-line placode and epibranchial placodes of IX and X. × 37.

Fig. 17 Frontal section, showing crest cells migrating over mesoderm of hyoid and branchial arches and above optic vesicles in stage 26–27. Also shows positions and relative thickness of VII and X lateral-line placodes and epibranchial placodes of IX and X. × 37.

Fig. 18 Frontal section, showing ophthalmic placode on right side lying close to anterior portion of neural crest migrating upon mesoderm of mandibular arch in stage 26–27. × 37.

Further rapid growth of neural crest has taken place ventrally, accompanied by a thinning out of the crest cells over the mid-dorsal portion of the brain (fig. 4). The anterior border of the eye is traversed as far as the dorsal border of the nasal placode by a narrow strip of loosely arranged cells. The mesoderm of the

mandibular arch is covered by crest cells to a level below the ventral border of the eye. Sections show that the crest cells are beginning to wrap around the mesoderm of the arch—a condition which can be seen more clearly in stage 30. The hyoid and first branchial crest-cell groups have extended farther ventrally to about the same level. The hyoid group (fig. 17) is quite thick and packed tightly against the arch. The first branchial group is more slender and curves slightly toward the hyoid arch. The remaining portion of the branchial mesoderm is being covered by a band of the neural crest which is slightly constricted at its union with the neural crest above. The posterior extension of the neural crest is diminished as its cells migrate toward the gill region, the extremity reaching only to a point above the posterior border of the second somite. It is from this posterior portion above the first three somites that the crest cells of the second, third, and fourth branchial arches have their source.

Placodes. Over the dorsal border of the eye the ophthalmic placode is a long narrow thickening which gives the appearance of having a broad contact with the loose neural crest against which it lies (fig. 18). The triangular placode anterior to the auditory placode approaches more closely the dorsal border of the crescent-shaped thickening above the hyomandibular cleft (fig. 4). The posterior margin of the placode of the lateral-line ganglion of VII is giving off a large number of cells which approach the neural crest lying along the anterior border of the auditory placode. The epibranchial placode of VII is situated immediately below and anterior to this cell proliferation as a very slight ectodermal thickening lying close to the lower border of the lateral-line placode. The epibranchial placode of IX lies in a level somewhat dorsal to its position in stage 25. On its inner surface loose cells containing mitotic figures lie closely against the crest cells descending on the anterodorsal margin of the first branchial arch, indicating that placodal cells are being given off to form a ganglion (fig. 17). Posterior to the placode which lies in the upper extremity of the first branchial cleft a somewhat elongated area of the ectoderm is slightly thickened. It is very probable, judging from the study of older stages, that these placodes are the

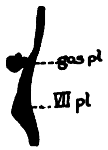
epibranchial placodes which contribute placodal cells to form the visceral sensory ganglion of X. The placode of the vagus lateral-line ganglion and of the midbody line is situated posterior to its former position and lies across the anterior border of the first somite above the neural crest of the second, third, and fourth branchial arches (fig. 17). In the ectodermal intersegment of the first and second somites is a prominent thickening, which later is the position of the placode which gives rise to the dorsal body line of sense organs and a portion of the vagus lateral-line ganglion.

Stage 30

Neural crest. This stage shows the rapid descent the neural crest has made in its progression ventrally. No longer are the crest cells from both sides continuous over the dorsal border of the brain (fig. 5). The crest cells over the mesoderm of the mandibular arch and over the dorsal and anterior borders of the eye have broken all connection with those of the hyoid arch. The maxillary and mandibular processes of the mandibular arch have become covered with crest cells. The manner in which the mesoderm of the visceral arches is becoming completely surrounded by a layer of crest cells two to three cells thick is shown in figure 19. The mesoderm of the mandibular and hyoid arches is completely surrounded by the crest cells, while the first branchial arch is enclosed around its anterolateral and posterior borders. The crest cells over the anterior border of the eye now extend as a narrow band to the dorsal border of the nasal placode. A few scattered crest cells are found along the dorsal border of the auditory vesicle which are continuous with crest cells lying on its anterior and posterior borders. A few crest cells come to lie below the ventral border of the auditory vesicle, due to the rapid ventral shifting of crest cells from above. The crest cells from the anterior border of the auditory vesicle continue with those of the hyoid arch, which have descended ventrally farther than the crest cells of the first branchial group. The dorsal region of the crest cells lying posterior to the auditory vesicle consists of loosely arranged cells which extend to the middle of

the first somite. All of the crest cells are then confined to the broad band which now descends over the mesoderm of the second, third, and fourth branchial arches.

Placodes. The ophthalmic placode is still an elongated thickening (fig. 20), in which may be seen mitotic figures. Its posterior border is in close contact with the neural crest. It gives



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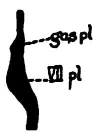

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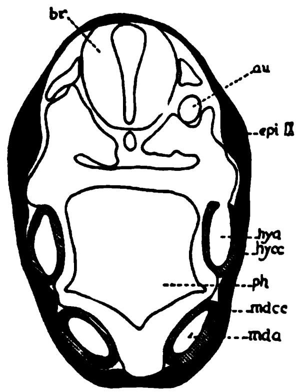

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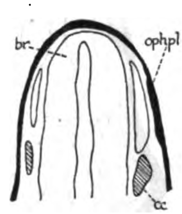

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Fig. 19 Cross-section, showing in stage 30 the manner in which crest cells are completely surrounding mesoderm of hyoid and mandibular arches. Also shows ectodermal thickening in dorsal portion of hyobranchial cleft containing epibranchial placode of IX. × 37.

Fig. 20 Frontal section of elongated ophthalmic placode in stage 30. × 37.

Fig. 21 Frontal section of stage 30, showing point of contact of gasserian ganglion near its distal end with a small ectodermal thickening at anterior extremity of VII lateral-line placode. × 37.

Fig. 22 Frontal section, showing in stage 28 a small ectodermal thickening (gasserian placode) slightly below the anterior extremity of VII lateral-line placode. × 37.

the appearance of contributing placodal cells to the ophthalmic ganglion.

Near the anterior extremity of the elongated placode of VII there is a slight thickening in the ectoderm. A few loose cells are connected with the inner side of the thickening (fig. 21). This area may be a small placode concerned with the gasserian portion of V. The only similar area in stages earlier than this

can be found in stage 28. There is a slight thickening in the ectoderm in front of and slightly below the anterior extremity of the VII placode (fig. 22). The placode of the lateral-line ganglion of VII has extended to the dorsal border of this placode, with which it fuses near the dorsal portion of the hyomandibular cleft.

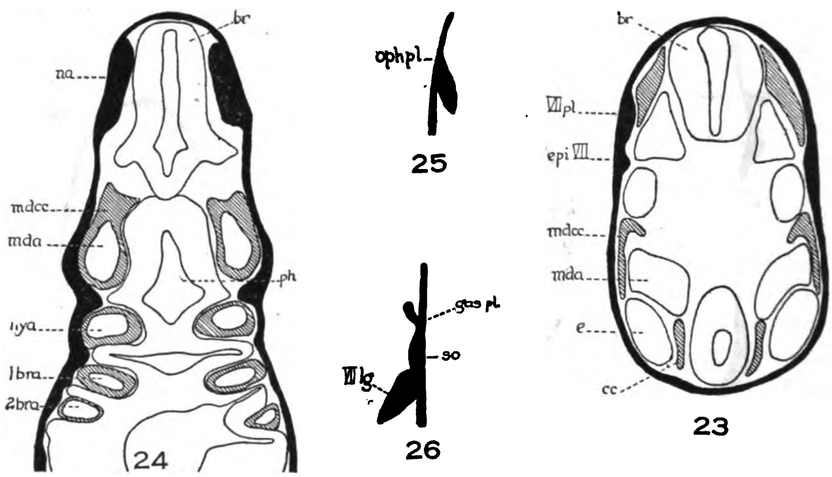

Fig. 23 Cross-section, showing in stage 30 the epibranchial placode of VII lying below lateral-line placode of VII and also crest cells descending over mandibular arch and dorsal portion of optic vesicles. × 37.

Fig. 24 Frontal section through lower portions of mesoderm of mandibular, hyoid, first and second branchial arches in stage 33, showing manner in which arches are being completely surrounded by crest cells. × 37.

Fig. 25 Frontal section, showing in stage 33 broad contact of ophthalmic ganglion with ectoderm. × 37.

Fig. 26 Frontal section, showing in stage 33 contact of distal portion of gasserian ganglion with an ectodermal thickening at anteroventral margin of supra-orbital primordium. × 37.

The posterior extremity of the VII lateral-line placode is contributing cells to a mass lying close upon the anterior border of the auditory vesicle. Many mitotic figures can be seen in this portion where the cells are separating from the placode. Above the point of fusion of the placode of VII with the placode near the dorsal portion of the hyomandibular cleft, the ectodermal thickening becomes broadened in the anterodorsal direction

(fig. 5). In the lower portion of the hyomandibular cleft, on a level with the ventral border of the optic vesicle, appears an elongated ectodermal thickening, where later primordia of the ventral hyomandibular and mandibular groups of lateral-line sense organs appear.

The epibranchial placode of VII can be seen as a small ectodermal thickening somewhat deeper than in stage 26–27, lying on the upper anterior border of the hyoid arch just below the lateral-line ganglion placode of VII (fig. 23). The epibranchial placode of IX appears in approximately the same position as in the previous stage and is preparing to give off a mass of cells (fig. 19). The vagus lateral-line placode has begun to give off a large mass of cells which comes in contact with a thin layer of the neural crest. Above the dorsal extremity of the first branchial cleft is the small epibranchial placode which is a visceral sensory placode of the vagus. In the region below the lateral-line ganglion placode of the vagus the ectoderm is somewhat thickened and represents the posterior portion of the visceral sensory placode of the vagus. In the intersegment of the first and second somites, the prominent ectodermal thickening appears somewhat larger than in stage 26–27.

Stage 33

Neural crest. The rapidly diminishing area of neural crest in the region over the dorsal portion of the eye is seen in this stage (fig. 6). The crest cells have passed farther behind the nasal placode on its posterior border where they join, along the ventral border of the optic vesicle, those which surround the maxillary process. The crest cells over the dorsal border of the hyoid arch are continuous with a broad area of crest cells which lie upon the anterior and ventral borders of the auditory vesicle. The mesoderm of the hyoid and first branchial arches is covered by crest cells which have now extended to the ventral borders of these arches. The crest cells lying above the remainder of the branchial region have diminished in number and now lie in a thin layer. The neural crest can no longer be found over the dorsal border of the auditory vesicle. The broad sheet of crest

cells which appeared in stage 30 over the posterior two-thirds of the branchial region has now split into two bands, the anterior of which is covering the second branchial arch, while the posterior shorter band lies on the mesoderm of the third and fourth branchial arches.

In the lower portion of the visceral arches (fig. 24) the mesoderm of the mandibular, the hyoid, and the first and second branchial arches can be seen on either side entirely surrounded by neural crest. The third branchial arch which appears in more dorsal sections is likewise entirely surrounded by neural crest. As the sections are followed ventrally, many mitotic figures can be seen in these rings of neural-crest cells, indicating a rapid increase in the number of cells as well as rapid migration. They have grown very abundant in their position around the visceral arches, especially so around the anterior portion of the mandibular arch where they are continuous ventrally in front of and behind the stomadaeum upon the maxillary and mandibular processes.

Placodes. The ophthalmic ganglion, at its anterior extremity, has a wide contact with the ectoderm (fig. 25). Mitotic figures appear on the ectodermal side of the contact throughout this and other sections. The extent of the contact is indicated in figure 6. The gasserian ganglion near its distal end has a definite contact, for about four sections, with the ectoderm at the anteroventral border of the VII lateral-line placode (fig. 26). This is possibly a placode for the gasserian ganglion. The actual contact is very brief and its history is difficult to follow. In later stages it is not possible to detect the placode. Many placodal cells forming the lateral-line ganglion of VII are splitting off from the posterior portion of the elongated placode which lies above the hyoid arch and hyomandibular cleft (fig. 6). The lower portion of the lateral-line ganglion, which is thus forming, consists of cells less compact at this stage. These later supply the hyomandibular division of the lateral-line component of VII. Where the VII lateral-line placode fuses with the placode in the dorsal portion of the hyomandibular cleft (fig. 6) the anterodorsal extension is larger than in stage 30. In the epibranchial placode of VII are

numerous mitotic figures showing a rapid development of placodal cells (fig. 27). It is difficult to mark off the upper portion of this placode because of its proximity to the ventral border of the loose cells of the lateral-line ganglion above. The primordium of the

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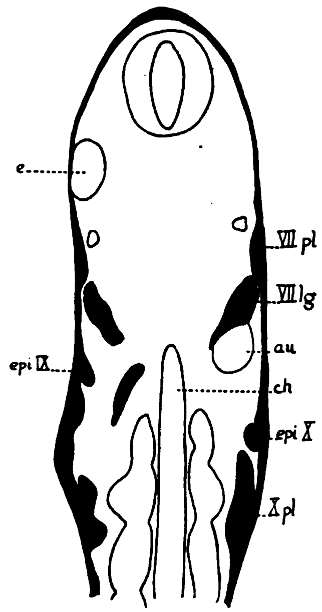

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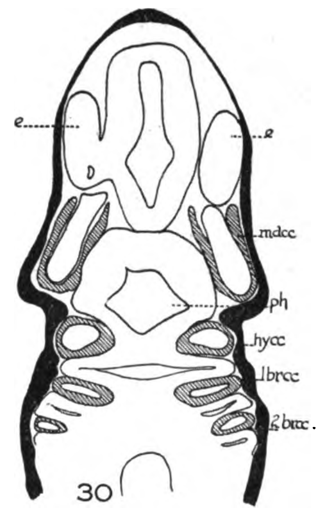

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Fig. 27 Frontal section, showing epibranchial placode of VII in stage 33 proliferating a large mass of cells. × 37.

Fig. 28 Frontal section, showing in stage 33 lateral-line ganglion and placode of VII, rounded epibranchial placode of IX on left side and epibranchial placode of X on right anterior to X lateral-line placode in process of forming ganglion. × 37.

Fig. 29 Frontal section, showing at stage 33 lateral-line placode of IX posterior to auditory capsule. × 37.

Fig. 30 Frontal section, showing at stage 35 crest cells surrounding mesoderm of mandibular, hyoid, first and second branchial arches and also increase in number of crest cells on median side of hyoid arch. × 37.

ventral hyomandibular line of sense organs in the lower portion of the hyomandibular cleft is somewhat elongated and deeper than in stage 30. The epibranchial placode of IX (fig. 28) is giving off a rounded mass of cells just above and slightly posterior

to the hyobranchial cleft. Above the epibranchial placode of IX there appears a small round thickening, possibly the IX lateral-line placode, in the ectoderm near the posteroventral border of the ear. It merges into thickened ectoderm which posteriorly is identified as the epibranchial placodal region of X (fig. 29). Above the first branchial cleft a placode is also giving off cells in large numbers which lie close to the neural crest. It is connected by loose cells which lie along the ventral border of the vagus lateral-line ganglion next to a thickened portion of the ectoderm (fig. 6). This entire mass of cells, including the placode in the dorsal portion of the first branchial cleft, is the placodal mass which gives rise to the visceral sensory ganglion of X. The anterior extremity of the elongated placode over the posterodorsal border of the crest cells of the branchial region is splitting off a large mass of placodal cells to form the vagus lateral-line ganglion (fig. 28). The placode in the intersegment of the first and second somites has increased somewhat in size.

Stage 35

Neural crest. The remains of neural crest over the dorsal portion of the eye exist only as a very narrow band of loose cells which are continuous anteriorly as far as the nasal placode, and posteriorly as far as the mandibular crest cells with which they are united (fig. 7). The posterior border of the nasal placode is covered by a group of crest cells continuous with the maxillary group, which in turn joins ventrally in front of the stomadaeum with the maxillary group of the opposite side. Only a few loose crest cells are still visible along the anterior border of the auditory vesicle. The crest cells extend to the ventral extremity of the mesoderm of the hyoid arch near the pericardium. Above the gill region and posterior to the auditory vesicle, a narrow longitudinal band of crest cells extends to near the posterior border of the first somite. Descending ventrally are bands of crest cells which supply each of the branchial arches. The recent splitting of the broad most posterior band of crest cells, which existed in stage 30 (fig. 6), is indicated by the ventral continuations of the crest-cell bands of the third and fourth branchial arches.

A few loose crest cells appear to join the ventral extremities of the second, third, and fourth branchial arches.

A new feature in the rapidly migrating crest cells now appears. They begin to accumulate along the median side of the arches where many mitotic figures can be seen, especially in the lower halves of the latter. The typical condition is seen in the section on the median side of the hyoid arch. At this level the mandibular arch is not completely surrounded by crest cells on its anterior border as it is farther ventrally. If the sections are studied still farther ventrally, it can be seen that the crest cells are continuous across the ventral extremities of the mesoderm of the hyoid arches in front of the pericardium and across the ventral extremities of the mandibular and maxillary processes behind and in front of the stomadaeum. If the sections are studied farther dorsally, all the branchial arches show, along their ventral halves, the condition similar to that of the hyoid arch (fig. 30).

Placodes. No longer is there any contact of the ophthalmic ganglion with the ectoderm, although its anterior end lies close to it. All traces of an ectodermal thickening in this region have disappeared, leaving the ectoderm the same thickness as before its appearance.

The primordium of the supra-orbital line of sense organs now appears as a prominent ovoid placode whose anterior end is directed anterodorsally (fig. 7). Its posterior border is joined by the incipient ramus ophthalmicus superficialis VII, while its ventral border is connected with the ectodermal thickening in the dorsal portion of the hyomandibular cleft. Along the course of the anterior extremity of the ectodermal thickening, which was shown in stage 33, there now descends near the posterior border of the optic vesicle a well-defined primordium of the infraorbital line of sense organs. Along the posterior extremity of the ectodermal thickening is the small primordium of the hyomandibular group of sense organs which is connected to the incipient ramus buccalis VII.

The epibranchial placode of VII lies just posterior to the primordium of the hyomandibular group of sense organs and upon the posterior portion of the hyomandibular cleft (fig. 7). It has

a broad contact with the distal end of the visceral sensory portion of VII extending dorsoventrally in the cleft. Many mitotic figures may be found at or near the point of contact, indicating that many placodal cells are still steadily being split off to form the ganglion.

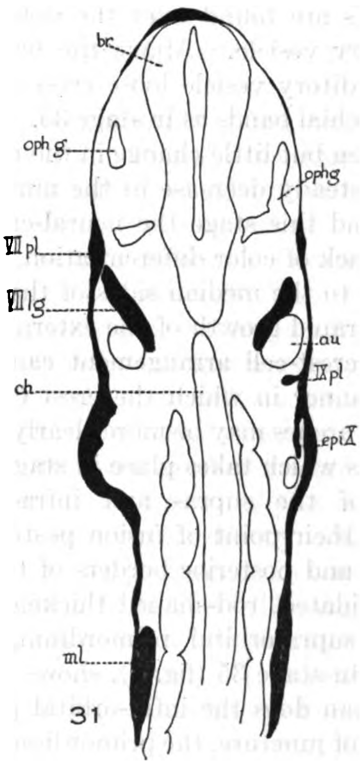
The lateral-line primordium in the lower portion of the hyomandibular cleft has increased in depth and at the same time has slightly lengthened. Its upper extremity lies along the posterior border of the anlage of the balancer (fig. 7).

The epibranchial placode of IX lies in the same position and still has a narrow contact with the placodal cells of the visceral sensory ganglion of IX. Over the first and second branchial clefts the epibranchial placodes of X, now continuous as a single elongated placode, are contributing large numbers of cells splitting off en masse to the visceral sensory ganglion of X lying ventrally to the vagus lateral-line ganglion (fig. 31).

Along the posterior border of the ear and slightly above the epibranchial placode of IX appears another rounded placode, which is the primordium of the anterior portion of the occipital group of lateral-line sense organs. It is in close contact with the IX lateral-line ganglion (fig. 31). Posterior to this and above the first branchial cleft is another rounded placode, which is the primordium of the posterior portion of the occipital group of lateral-line sense organs.

In the dorsal portion of the intersegmental ridge of the first and second somites is a rounded placode (fig. 7) connected by placodal cells from its anteroventral margin to the lateral-line ganglion of X, indicated by dotted line. This is the primordium of the dorsal body line of lateral-line sense organs. On a level with its ventral border an elongated club-shaped placode extends from the anterior border of the second somite to near the posterior border of the third somite. This is the primordium of the midbody line of sense organs and is connected by a small strand of cells at its anterior border to the posterior border of the lateral-line ganglion of X. Just below the anterior extremity of the primordium of the midbody line is a small rounded placode lying close to the posterior extremity of the lateral-line ganglion

near the dorsal extremity of the fourth branchial arch. This is the primordium of the ventral body line of sense organs. It is connected by a few placodal cells from its dorsal border to the vagus lateral-line ganglion.



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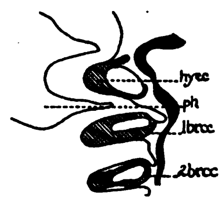

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Fig. 31 Frontal section, showing at stage 35 the IX lateral-line ganglion and placode anterior to epibranchial placode of X. Farther posteriorly is mid-body-line primordium. × 37.

Fig. 32 Frontal section, showing at stage 36 aggregation of crest cells on median surfaces of mesoderm of hyoid, first and second branchial arches. × 37.

It is at this stage in the developing embryo that all of the primordia of the lateral-line groups of sense organs are laid down in visible form in the ectoderm.

Stage 35+

Neural crest. With the exception of a more compact arrangement in the branchial and hyoid regions, there is but little change in the neural crest over the mesoderm of the branchial and hyoid arches (fig. 8). No crest cells are found over the dorsal and anterior borders of the auditory vesicle. Above the branchial arches and posterior to the auditory vesicle loose crest cells lie in the same relation to the branchial bands as in stage 35. In the mandibular region there has been but little change in the relation of the crest cells except for a steady decrease in the number of cells dorsal to the eye. Beyond this stage the neural-crest arrangement is obscured by the lack of color differentiation, by the further migration of crest cells to the median sides of the mesoderm of the arches and by the rapid growth of the external gills, so that further knowledge of crest-cell arrangement cannot be gained by dissection. The manner in which the crest cells assume their positions around the arches may be more clearly shown in the description of the process which takes place in stage 36.

Placodes. The primordia of the supra- and infra-orbital lateral-line sense organs, from their point of fusion posterior to the eye, curve over the dorsal and posterior borders of the eye, respectively, as narrow, consolidated, rod-shaped thickenings in the ectoderm (fig. 8). The supra-orbital primordium, when compared with its appearance in stage 35 (fig. 7), shows a more decided change in its shape than does the infra-orbital primordium. Posterior to their point of juncture, the primordium of the hyomandibular lateral-line sense organs extends ventrally along the upper portion of the hyomandibular cleft as a short rod similar in character to that of the supra- and infra-orbital primordia. The primordium of the ventral hyomandibular group of lateral-line sense organs now extends farther ventrally from the posterior border of the base of the balancer in the lower portion of the hyomandibular cleft and its rod-shaped placode has somewhat increased in depth. In addition to the primordia shown in the previous stage (fig. 7), there is now a primordium of the mandibular group of lateral-line sense organs extending as a short ectodermal thickening along the ventral border of the base of

the balancer fused with the dorsal extremity of the ventral hyomandibular primordium.

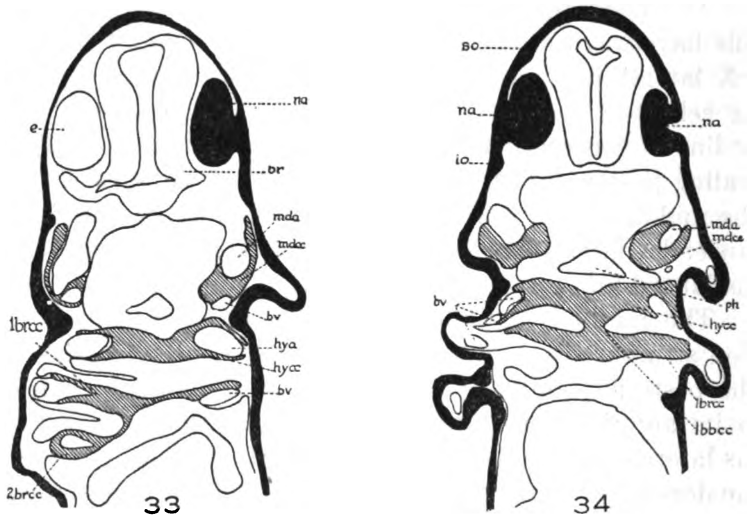
The epibranchial placode of VII still persists in contact with the visceral sensory ganglion of VII where many mitotic figures can be seen. The epibranchial placodes of IX and X still persist and a few cells appear to be splitting off to the visceral sensory ganglia.

The two primordia of the occipital group of lateral-line sense organs have increased in size and their close contact to the IX and X lateral-line ganglia gives them the appearance of contributing cells to those ganglia. The primordium of the dorsal body line of sense organs now appears as an elongated placode extending posterodorsally from the middle of the second somite to the middle of the third somite, connected at its anterior end by a slender lateral-line nerve to the posterior extremity of the vagus lateral-line ganglion whose position is indicated by dotted line. The midbody lateral-line placode is an elongated club-shaped structure extending from the middle of the fourth somite to the posterior border of the seventh somite. It is connected from its anterior border by a slender lateral-line nerve to the vagus lateral-line ganglion in common with the lateral-line nerve of the dorsal body line. The ventral body line is represented by a small ovoid placode which lies on the posterior border of the fourth branchial arch. It is connected dorsally to the posterior portion of the vagus lateral-line ganglion by a small lateral-line nerve.

Stage 36

Neural crest. Since stage 35, there has been a more rapid migration of the crest cells to the median border of the arches where they accumulate in large numbers (fig. 32). As one studies the sections farther ventrally, this becomes more apparent, and it can be seen that the process is carried on at the expense of the crest cells on the lateral, anterior, and posterior borders of the arches where they lie in a thin layer, or where, in the case of the lateral borders, they are occasionally entirely lacking. Within the aggregation of crest cells on the median borders of the arches

may be seen many mitotic figures indicating that there still is a rapid increase in the number of cells as well as a migration. Up to this point the crest cells have shown characteristic fine yolk granules in their cytoplasm which was an aid in differentiating them from the surrounding tissue. Now the yolk granules have become very small or have been largely absorbed within the crest-cell aggregations. In many places the mesoderm of the branchial



Figs. 33 and 34 Frontal sections, showing at stage 36 and 37–38, respectively, large numbers of crest cells at median and posterior borders of mesoderm of mandibular arch and fusion in the midline of crest cells of hyoid and first branchial groups to form the first basibranchium. × 37.

arches is being hollowed out by the formation of developing blood vessels.

If the sections are followed ventrally it can be seen that the crest cells of the hyoid arches unite in the midventral line in front of the pericardium. The first branchial group, after it has united with the second branchial group (fig. 33), joins the crest cells of the first branchial group and from the opposite side attempts farther ventrally to continue with the posterior border of the fusion of the hyoid crest cells. The third and fourth branchial crest-cell groups have not yet joined at their ventral extremities.

As one studies the ventral portions of the mandibular and maxillary processes, it is evident that there is an abundance of crest cells, especially around the former (fig. 33).

Placodes. The supra-orbital primordium now extends as far ventrally as the dorsal border of the olfactory organ (fig. 9), while the infra-orbital descends to the posterior border of the olfactory organ. The hyomandibular primordium has now joined the dorsal extremity of the ventral hyomandibular primordium posterior to the balancer. The mandibular primordium extends anteroventrally, then mesially to the region of the lower jaw. The ventral hyomandibular primordium extends farther ventrally, then mesially where it begins to take an anterior direction upon the ventral surface of the lower jaw. Posterior to the auditory vesicle, four separate placodes now mark the positions of the primordia of the occipital group of lateral-line sense organs. The two anterior placodes of this group lie close to the posterior border of the ear, while the two posterior lie above the base of the third external gill; the more ventral of the latter is much elongated in the anteroposterior direction.

The dorsal body-line primordium has extended as far as the seventh somite, followed by a thin lateral-line nerve upon which the anlagen of two sense organs have appeared. The primordium of the midbody line is a very much elongated placode which extends over about seven and one-half somites as far as the sixteenth somite. Along its lateral-line nerve appear the anlagen of five sense organs. The ventral body line can be seen descending behind the external gills anterior to the limb region connected by a slender lateral-line nerve.

The epibranchial placode of VII still has a slight contact with the distal end of the visceral portion of VII. No other epibranchial placodes are found to be giving off cells to the ganglia at this stage.

Stage 37–38

Neural crest. The aggregations of crest cells along the median borders of the visceral arches now show a more compact arrangement. So dense is the tissue that it appears to be composed

mainly of nuclei with but little cytoplasm. The density increases as one studies the sections farther ventrally. The few yolk granules which are present are similar in size to those of the smaller ones in the mesoderm of the arches and many mitotic figures can still be seen among the crest cells. The fusion of the median ends of the first branchial groups with those of the hyoid in the midventral line furnishes an illustration of the more compact arrangement of the cells (fig. 34).

As one follows the sections dorsally, the median ventral extremities of the branchial groups on each side are seen to approach each other at successive levels. It is now quite obvious that the branchial groups have taken on the form of the early branchial cartilaginous skeleton. The dorsal extremities of the branchial groups are continuous with loose crest cells, which possibly linger behind to form connective tissue, although the rapid differentiation of the surrounding mesoderm prevents any accurate determination of this. Contiguous to the neural-crest aggregations the blood-vessel spaces can be seen permeating the mesoderm of the arches, the circulation of blood in the mesoderm of the arches having been established in stage 36–37. The crest cells are more loosely arranged around the periphery of the mandibular arch—a possible indication that they are giving rise to connective tissue. Over the maxillary process the crest cells are followed with great difficulty owing to the fact that in front of, as well as behind the stomadaeum there are many loose mesenchyme cells. The crest cells have already made their way into the balancer, as has been shown by Harrison ('21).

Placodes. Since the epibranchial placodes no longer appear in the ectoderm, further description of the placodes will be confined to the primordia of the lateral-line system (fig. 10). The supra- and infra-orbital primordia extend farther ventrally, passing respectively, around the anterior and posterior borders of the nasal organ. They then pass medially in anticipation of the formation of the sense organs on the upper jaw around the external nares. The hyomandibular primordium has increased slightly in length and passes to near the posteroventral border of the balancer. The mandibular and ventral hyomandibular primordia have extended

further medially than in the previous stage described. The occipital group is still represented by four primordia, which are becoming elongated as they approach each other. The dorsal body line has progressed posteriorly as far as the twelfth somite, along the path of which have appeared the anlagen of a varying number of sense organs. The midbody line has progressed caudally as far as the twenty-seventh somite. As it reaches a point just above the anal region it curves dorsally a short distance, then caudally where it travels along the upper border of the somites of the tail. Along its course have developed many anlagen of sense organs. The ventral body line has descended behind the external gills anterior to the limb region to the ventral border of the mesoderm of the limb bud where it begins to bend caudally.

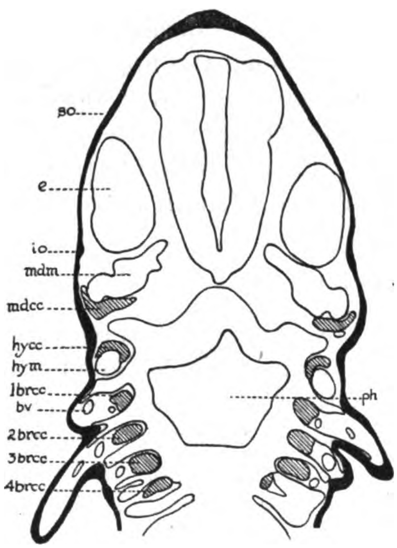
Stage 39

Neural crest. Since the previous stage the aggregations of neural crest have become still more compact (fig. 35) and may now be regarded as the procartilage of the visceral skeleton. Only a few crest cells can be identified outside of the procartilage region, although, as has already been suggested, many of the loose cells around the maxillary and mandibular groups may be of crest-cell origin and may be forming connective tissue. The procartilage still contains many small yolk granules and also many mitotic figures. The position of the mesoderm of the branchial arches is now occupied by blood vessels and anlagen of branchial muscles, while in the position of the mandibular and hyoid mesoderm are the anlagen of mandibular and hyoid muscles.

Stage 42

Neural crest. In stages 40–41, of which no sections have been figured, the only perceptible changes in the procartilage are the gradual disappearance of yolk granules, the decrease in the number of mitotic figures, and the steady increase in the density of the cellular arrangement. In stage 42 true cartilage is laid down as well-defined, solid bars in the positions formerly occupied by the procartilage (fig. 36). The yolk granules have entirely disappeared from the branchial cartilages.

A study of the early branchial skeleton up to this stage shows conclusively that so far the cartilages have their origin in the neural crest. However, in the case of the second basibranchial, which is the last cartilage of the visceral skeleton to appear, stage 42 shows more convincingly than any of the previous stages that this cartilage is of mesodermal origin. The caudal extremity of



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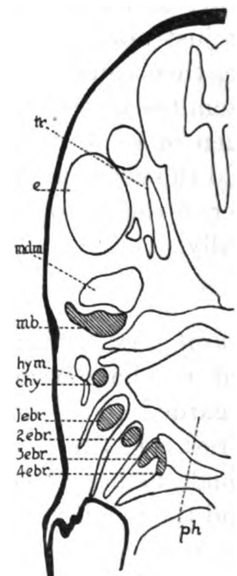

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Fig. 35 Frontal section through mesoderm of visceral arches at stage 39, showing positions of their procartilages. × 37.

Fig. 36 Frontal section through the cartilages of the arches at stage 42 when true cartilage is laid down, showing the same positions of the cartilages occupied by aggregations of crest cells. × 37.

the first basibranchial is attached to mesoderm which continues ventrally toward the pharynx. Out of this mesoderm along the anterior part of the pericardial chamber is formed the second basibranchial. Its distal half lies ventral to the level of the other cartilages. The mesoderm continuous with its distal extremity is the anlage of the thoracicohyoideus muscles, while the mesoderm continuous with its proximal or attached extremity is the anlage of the geniohyoid muscles. It has not developed cartilage

cells at this stage and its large characteristic mesodermal yolk granules (fig. 37), which are retained for a long time, make a sharp contrast with the other branchial cartilages, which now contain practically no yolk granules.

Stage 46+

Lateral-line groups of sense organs. The subsequent growth of the lateral-line primordia is merely a matter of the development

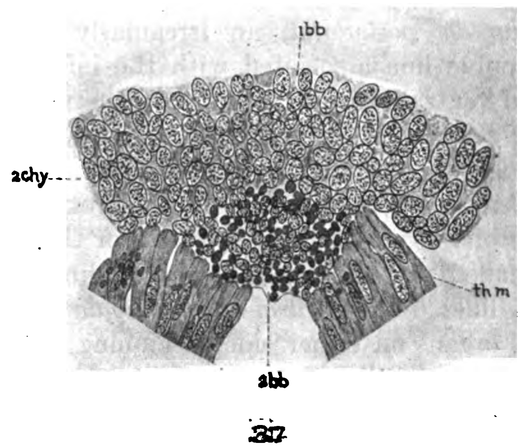


Fig. 37 Frontal section at the level of first basibranchium, showing at stage 42 formation of second basibranchium from mesoderm near anterior wall of pericardial chamber. × 250.

of sense organs out of the ectodermal thickenings as they are laid down in stage 37–38 (fig. 10).

The general distribution of the lateral-line sense organs has been described in a number of amphibians. Descriptive figures are shown by Kingsbury ('95) of the distribution of different groups of sense organs connected with the lateral-line system in eight groups of amphibians. However, two figures (figs. 11 and 12) have been included in this paper to show the groups of sense organs developed from the primordia of stage 37–38 as they are finally laid down in a 13½-mm. larva of Amblystoma punctatum.

The groups of sense organs may be described in the following manner: 1) *the supra-orbital,* a group usually arranged in a double line passing dorsal to the eye then ventral to a region median to the external nares; 2) *the infra-orbital,* a group sometimes in a single, sometimes in a double row, passing ventral to the eye to a region on the upper jaw ventral to the external nares; 3) *the occipital,* an approximately triangular group of variously arranged organs with a short line of four or five sense organs extending caudally as far as the region above the third external gill; 4) *the hyomandibular or postorbital,* an irregularly arranged group, usually a double line associated with the infra-orbital passing caudally and ventrally from the latter to the ventral side of the head; 5) *the mandibular,* a single line of sense organs extending along the side of the lower jaw from the symphysis back to the ventral extremity of the hyomandibular group and giving off, at the angle of the jaw, a branch of two or three sense organs, sometimes called the angular group, extending dorsally to the infra-orbital line; 6) *the ventral-hyomandibular group,* a double line of sense organs on either side, beginning at the symphysis of the jaw passing median to the mandibular group to meet the latter at its posterior extremity, where it gives off a single line of seven or eight sense organs which passes directly mesially, but does not join with the corresponding branch of the opposite side; 7) *the midbody line,* a single line of sense organs extending from a region above the proximal end of the limb behind the external gills to the region above the level of the anus, where it bends upward to pass along the upper border of the somites to the end of the tail; 8) *the dorsal body line,* a single line of sense organs extending from behind the dorsal end of the third gill along the upper border of the somites, where it joins the midbody line in the region above the anus; 9) *the ventral body line,* a single line of sense organs curving around the ventral border of the fore limb and extending to the ventral border of the hind limb.

In this morphological study, in which critical stages have been described, it is obvious that at the time of the closure of the neural folds (stage 21) no definite placodes can be accurately located, unless it be a slight indefinite thickening dorsal to the

eye. The ectodermal thickening over the dorsal extremity of the hyomandibular cleft as well as the longitudinal ectodermal thickening seems to have no significance except in so far as it is the result of a change in contour of the underlying tissue. As early as stage 25 (fig. 3), however, the placodes of lateral-line ganglia of VII and X can be identified, while all the epibranchial placodes can be located at stage 26–27 (fig. 4). The primordia of all the groups of the lateral-line sense organs are established at stage 35+ (fig. 8), and out of this pattern the sense organs are laid down in the skin.

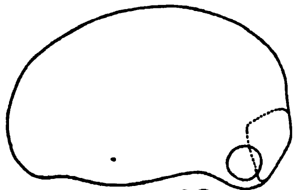
EXPERIMENTAL

All the operations were confined to the right side of the embryos and consist of two groups: *a*) extirpating the placodes in the ectoderm, followed by grafting into the wound indifferent ectoderm taken from another animal of the same age and, *b*) removing the neural crest either by scraping it loose or by removing it along with the upper half of the neural canal from the anterior border of the third somite to the anterior extremity of the brain above the eye.

Where indifferent ectoderm was transplanted into denuded areas the grafts were removed from animals which had previously been stained in the jelly capsule with Nile-blue sulphate (Detwiler, '17). This made possible daily observations upon the early growth and position of the implanted tissue.

The mortality of operated animals was lowered by keeping them during the three or four days following the operation in a glass chamber, around which cool tap-water ranging from 11° to 15°C. was constantly running. This retarded wound healing at first, but no disturbances were observed to affect the results of the experiments.

In stage 25 the auditory placode becomes marked externally as a small round pigmented spot a little below an elevation in the region of the hind-brain (fig. 3). This may be used almost invariably as a landmark in orienting operations upon the placodal regions in this and early embryos. Since there is no external evidence of the pigmented spot previous to this stage, the eleva-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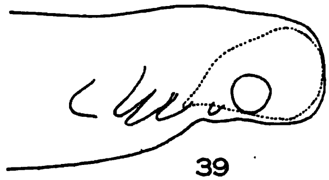
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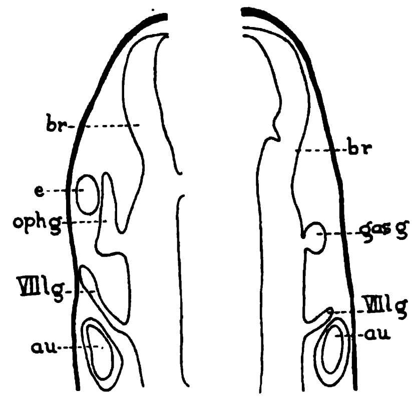

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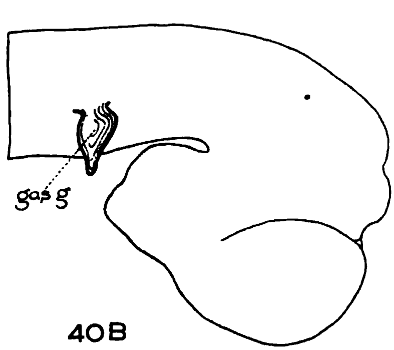

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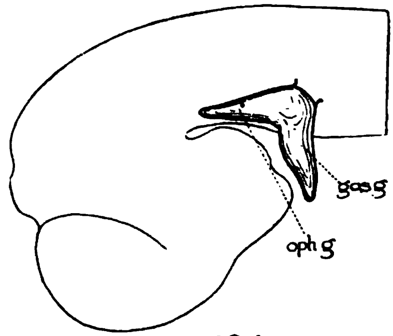

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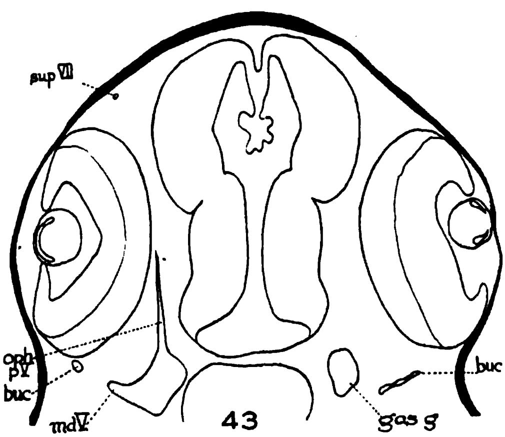

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tion in the region of the hind-brain lends itself as a landmark in orienting similar operations upon embryos from the closure of the neural folds to stage 25 (figs. 1 and 2).

A. *Extirpation of placodes*

1. *Removal of ophthalmic placode.* A triangular piece of ectoderm, including skin dorsal to the optic vesicle, was removed in the manner shown in figure 38 and indifferent ectoderm was grafted upon the denuded area. Such areas of ectoderm were taken from embryos varying in stages from 23 to 26.

A typical picture of the extension of the implanted blue ectoderm may be obtained from figure 39. Gradually the blue area extends ventrally until it includes the ectoderm over the eye and the regions anterior and posterior to it, always showing the more extensive migration towards the ventral half of the mandibular arch and towards the first external gill. As a result of this, the blue ectoderm several days after the operation comprises an area far greater in extent than the original extirpated area. After seven or eight days the Nile-blue sulphate gradually disappears and the transplanted area can often be located by its scarcity of pigment—a characteristic of the ventral ectoderm of older embryos. The same condition of the extension of blue implanted ectoderm may be seen in many other cases (figs. 48, 49, 55, 63). This may be largely due to a migration of the super-

Fig. 38 Showing triangular piece of ectoderm, indicated by dotted line and middorsal line which was excised in order to remove the ophthalmic placode at stage 23. × 10.

Fig. 39 Camera-lucida drawing of the same individual a few days after operation, showing extent of migration of implanted ectoderm stained in Nile-blue sulphate. × 10.

Fig. 40 a and b Showing, respectively, lateral views of a reconstruction in the trigeminal region of the normal and operated sides. Only a small gasserian ganglion appears on the operated side. × 50.

Figs. 41 and 42 Frontal sections of the specimen reconstructed in figures 40a and b comparing the normal and operated sides at levels of the root of V. × 37.

Fig. 43 Frontal section of specimen killed nineteen days after operation, showing on right side only the gasserian portion of V after the ophthalmic placode has been excised. × 37.

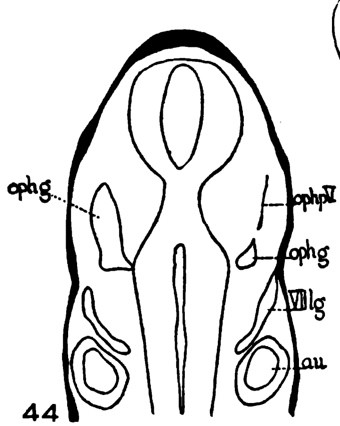
ficial cells of the ectoderm, for the deeper-lying sense organs in the intruded area are undisturbed. However, this is a subject for further investigation.

A specimen killed eight days after operation shows a complete absence of the ophthalmic ganglion and the ophthalmicus profundus V nerve (figs. 40 a and b). The size of the ophthalmic ganglion of the normal side of this individual is very large (fig. 41), and a comparison between it and the region of the root of the V on the operated side (fig. 42) renders a striking picture. The crest cells concerned in the formation of the visceral skeleton along the region of the mandibular arch are approximately normal in amount. This condition indicates that if any of the crest cells had been disturbed there has been a regeneration, and ample opportunity has been offered to contribute to the formation of the ganglion if such were their function. This individual also shows a diminution in the size of the gasserian ganglion as illustrated by figure 40 a. Another individual killed nineteen days after a similar operation at stage 23 shows no ophthalmicus profundus V nerve and the small ganglionic mass which does appear is apparently concerned only with the gasserian portion (fig. 43).

An individual which had been operated upon at stage 26 shows nine days later a very small ganglion represented by possibly no more than ten cells (fig. 44). The only representative of the ophthalmicus profundus V nerve appears in but one section and extends anteriorly as a slender nerve fiber. During stage 26 cells are being given off from the ophthalmic placode, and it is quite possible that a few of the placodal cells remained in the wound and later gave rise to the remnant of the ganglion which appears in this section. The gasserian ganglion is also smaller in this individual than is the normal ganglion of the left side.

An individual which was operated upon at stage 23 shows a small ophthalmic ganglion in an abnormal position (figs. 45 a and b). This ganglionic mass lies median to the eye and somewhat dorsal to the optic nerve. It is connected to the brain by a slender group of fibers which pass back through the gasserian ganglion into the brain. At the lower anterior portion of this

ganglionic mass there arises a slender nerve which sends a few cutaneous fibers to the skin over the anterodorsal portion of the eye. It then follows over the dorsal border of the nasal organ, in the region of which it gives off cutaneous fibers. In the distribution of its fibers this nerve issuing from the anteriorly placed ganglion simulates the ophthalmicus profundus V nerve. A



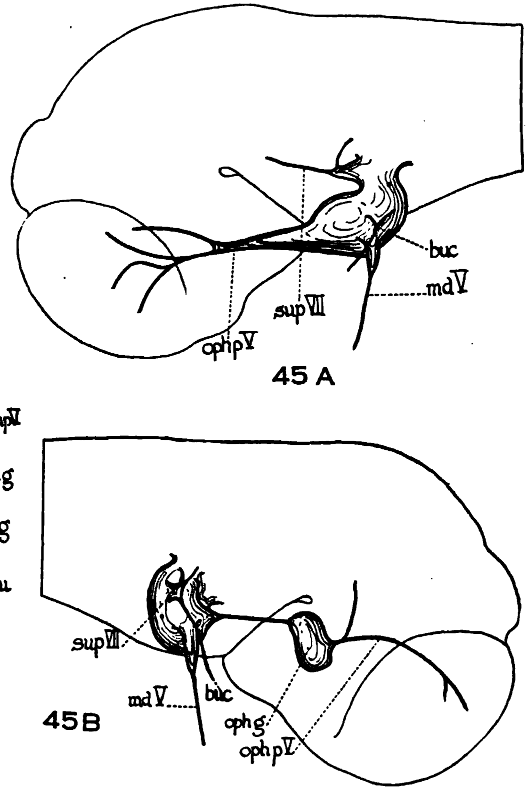


Fig. 44 Frontal section, showing relative difference in size of the ophthalmic ganglia on normal and operated sides. Part of the ophthalmic placode was possibly not removed. × 37.

Fig. 45 a and b Lateral views of a reconstruction, showing, respectively, normal and operated sides in the region of V and VII. The posterior portion of VII ganglion is not shown. The operated side (fig. 45 b) shows a small ophthalmic ganglion displaced anteriorly and lying above the optic nerve. It is connected posteriorly with the V and VII complex by a long slender root.a few fibers from its dorsoposterior border. A small ophthalmicus profundus V nerve extends from its anteroventral border. × 50.

study of the records of the growth of the transplanted blue ectoderm of this individual shows that not all of the ectoderm concerned in the formation of the ophthalmic ganglion was eliminated from the ectoderm in the region anterodorsal to the optic vesicle.

A control operation was made on several specimens in which the ectoderm was excised as in the usual operative procedure and replaced and allowed to heal into its normal position. When these specimens were sectioned they showed perfectly normal ophthalmic ganglia, indicating that if there had been any disturbance of the crest cells in the trigeminal region during the operation it did not, in itself, affect the formation of the ganglion.

A large number of individuals in which the excised area containing the preauditory placode and supra-orbital primordium was removed along with some of the ectoderm above the optic vesicle, show a similar displacement of the ophthalmic ganglion. Among these cases where the embryos were preserved within a few days after the operation ganglionic masses of cells lie close to the ectoderm. Their posterior ends become attenuated and no connection to the brain is discernible. The older individuals which were sectioned show cutaneous fibers issuing from the ganglionic mass comparable in their distribution to the fibers from the ophthalmicus profundus V nerve as in the case already cited (fig. 45 b).

It seems quite evident from the above results obtained that the formation of the ophthalmic ganglion is largely if not entirely dependent upon the placode in the ectoderm above the optic vesicle.

2. Removal of gasserian placode. A rather extensive rectangular piece of ectoderm was removed, including all the ectoderm around the dorsoposterior quadrant of the eye. An incision was made beginning at a point on the hyoid arch at a level with about the middle of the eye and passing dorsally some distance in front of the auditory placode to a level about the middle of the latter. The incision was then extended anteriorly parallel with the middorsal line to a point above the middle of the eye. From this point it was carried ventrally to a little above the middle of the

eye, from which it was extended posteriorly to the incision on the hyoid arch. The ectoderm thus outlined was very carefully removed and the wound was covered by a graft of blue indifferent ectoderm.

A specimen killed six days after operation at a time when the ophthalmic and gasserian ganglia have just fused at the point of the entrance of the trigeminal root into the brain shows on the operated side a somewhat smaller gasserian ganglion. The frontal sections show that the ganglion in its anteroposterior diameter varies little in the region near its root from that on the normal side, but as the ganglion is followed ventrally it soon becomes attenuated in the region near the upper posterior border of the eye, while on the normal side the mandibular nerve does not appear until the ganglion reaches the level with the middle of the posterior border of the eye. The diminution in the size of the gasserian ganglion is therefore represented mostly by a shorter dorsoventral axis. A large portion of the ophthalmic ganglion is displaced and lies near the anterodorsal portion of the eye. A group of fibers connect it to ganglionic cells which lie on the anterodorsal border of the gasserian ganglion. The crest cells over the mandibular arch are apparently normal in amount.

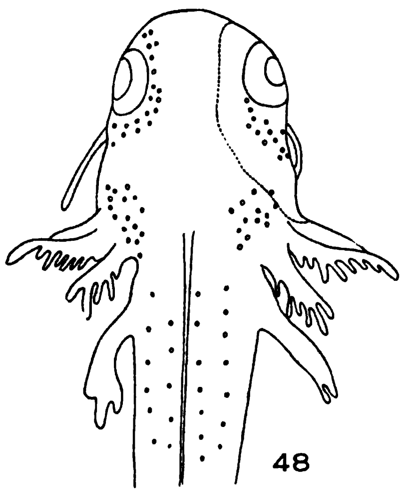
Two other specimens killed the same number of days after operation show the same results. In these cases, however, there appears to be a slightly smaller number of crest cells over the mesoderm of the mandibular arch.

A control operation was also made on several specimens, in which the ectoderm was excised in the usual manner and then replaced and allowed to heal in its normal position. Sections of these specimens show perfectly normal gasserian and profundus ganglia.

It has already been shown (figs. 40 b and 43) that when the ophthalmic placode was removed, including considerable ectoderm from the posterodorsal region of the eye, the gasserian ganglion was smaller than on the normal side. Among the cases, described in another section of this paper, in which placodes of VII were removed there appear two cases in which there is apparently no gasserian ganglion present (fig. 56 b).

It seems quite evident from these results that the ectodermal adhesion of the early gasserian ganglion is in the nature of a small placode which contributes cells to the formation of that ganglion.

3. Removal of preauditory placode and the supra-orbital primordium. In this type of operation the rectangular area of



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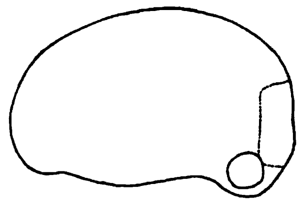

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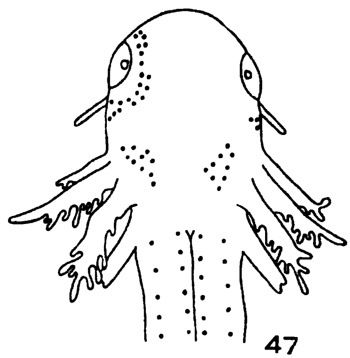

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Fig. 46 Camera drawing showing area of ectoderm excised in removing preauditory placode and supra-orbital primordium. Outlined by means of dotted lines and the middorsal line. × 10.

Fig. 47 Camera drawing, showing absence of the supra-orbital group of sense organs after removal of an area of ectoderm as shown in figure 46. × 10.

Fig. 48 Camera drawing, showing only three or four supra-orbital lateral line sense organs when not all of the early preauditory placode is removed during stage 21. The lightly pigmented area of transplant of indifferent ectoderm at time animal was killed is shown by dotted line. × 10.

ectoderm excised is outlined in figure 46. Indifferent ectoderm from the ventral side of another animal of the same age which had been previously stained with Nile-blue sulphate was grafted into the wound. This operation was confined to stages under stage 25.

Due to the inability to locate exactly the position of the auditory placode, especially in stage 21, it was often included wholly or in part in the excised ectoderm. Among such cases where the auditory vesicle was not entirely absent, it appeared as a small rudimentary vesicle near the skin.

An examination of specimens which were treated in the manner just described shows in eight cases no supra-orbital group of lateral-line sense organs (fig. 47), and consequently no ophthalmicus superficialis VII nerve. The operations in seven of these cases were performed at stage 23 and in one case about stage 21. The auditory vesicle appears as a rudiment in six cases, while in the other cases it is entirely absent. The larger portion of the lateral-line ganglion of VII which supplies nerve fibers to the supra- and infra-orbital groups of sense organs is represented in these cases by a very small ganglion. The infra-orbital line of sense organs with its corresponding nerve was never absent in this type of operation and it received its fibers from the small remaining portion of the above-mentioned lateral-line ganglion.

In a few cases where a small portion of the placode was, presumably, not entirely extirpated, the VII lateral-line ganglion is smaller than on the normal side and a small line of sense organs is the only representative of the supra-orbital line (fig. 48). In some cases a sensory line was represented by small, poorly developed organs and the ophthalmicus superficialis VII is so slender that it can be followed with great difficulty and then only when most favorably stained. Such cases show diminution in the size of the VII lateral-line component which supplies the supra-orbital and infra-orbital sensory lines. Throughout all these cases in which the supra-orbital sensory line is represented in part or in its entirety the ear is present and apparently normal in size.

The hyomandibular, ventral, and mandibular groups of lateral-line sense organs were never disturbed when such an area of ectoderm was removed. The VII lateral-line component of the ganglion supplying nerves to these groups was always normal. It is quite apparent that no other portions of the VII ganglion are lacking.

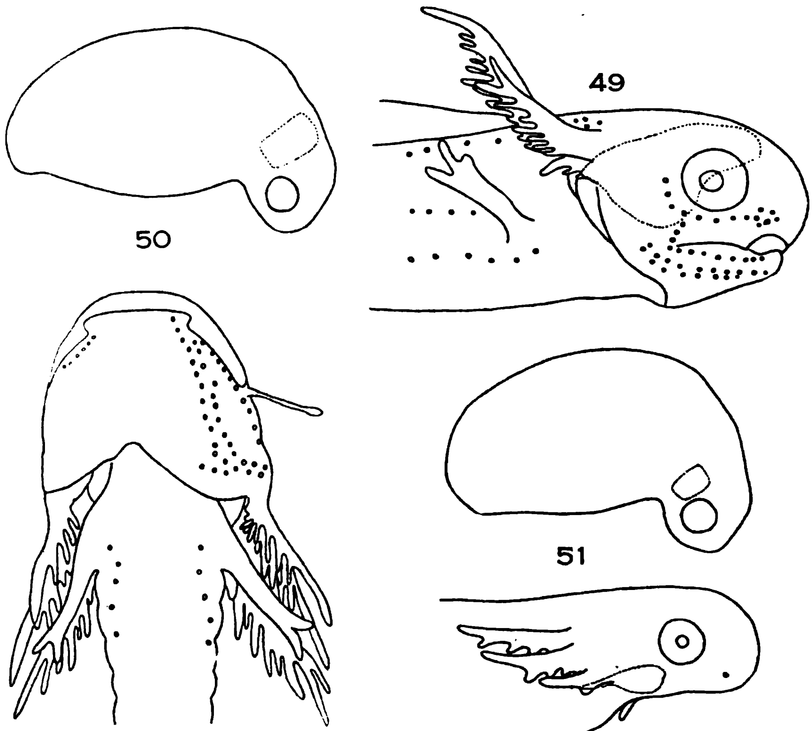


Fig. 49 Camera drawing, showing absence of hyomandibular group of sense organs after removal of an area of ectoderm shown in figure 50. × 10.

Fig. 50 Camera drawing, showing by dotted line an area of ectoderm removed at stage 26 in which was included the primordium of the hyomandibular group of sense organs as well as epibranchial placode of VII and supra-orbital primordium. × 10.

Fig. 51 Camera drawing, showing by dotted line an area of excised ectoderm in which was included the primordium of the ventral hyomandibular group of sense organs and part of the primordium of mandibular group. × 10.

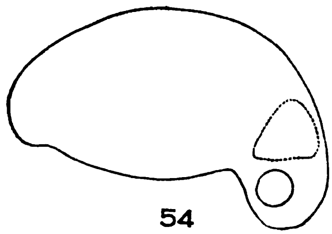
Fig. 52 Camera drawing, showing by dotted line position of implanted blue ectoderm a few days after operation in same individual shown in figure 51. The balancer is absent on operated side. × 10.

Fig. 53 Camera drawing twenty-one days later of the ventral side of embryo shown in figure 51. On the operated side there is only a small portion of the mandibular group of sense organs, while the ventral hyomandibular group is entirely absent. × 10.

4. Removal of the infra-orbital, hyomandibular, ventral hyomandibular and mandibular primordia. A number of trials were made upon embryos under stage 26 to remove separately, if possible, the primordia of the infra-orbital and hyomandibular groups of lateral-line sense organs on the side of the head by excising small segments of ectoderm behind the upper posterior border of the optic vesicle. These attempts proved to be fruitless, for in all cases the ingrafted tissue failed to suppress their development and complete regeneration of the two primordia took place. However, among the cases where an area of ectoderm including placodes of the VII ganglion was removed there appears one case, operated upon at stage 26, in which the hyomandibular group of sense organs is entirely absent, although the infra-orbital group is intact as well as the groups of sense organs on the lower side of the jaw (fig. 49). The area removed (fig. 50) also includes the epibranchial, the preauditory, and supra-orbital placodes. This seems to indicate that the infra-orbital and hyomandibular groups have separate primordia.

A few operations were made in an attempt to remove the primordia of the sense organs on the under side of the jaw. The most successful case appears in an individual operated upon at stage 25. The area removed is shown in figure 51, one day after operation, and it includes a small amount of ectoderm anterior and posterior to the lower portion of the hyomandibular cleft. Several days after operation (fig. 52) the area of the transplanted indifferent ectoderm may be seen at a lower level than its original position and the migration of its posterior portion extends into the anteroventral border of the first external gill. The balancer is lacking on this side. Twenty-one days after operation this individual reveals no ventral hyomandibular group of sense organs on the right side of the ventral portion of the lower jaw (fig. 53). However, there is a remnant of the mandibular group in the form of a few small sense organs along the anterolateral border of the lower jaw. This may possibly be an indication of the existence of a separate primordium for the mandibular group of sense organs.

5. Removal of the epibranchial placode of VII and surrounding ectoderm. The area of the extirpation in this type of operation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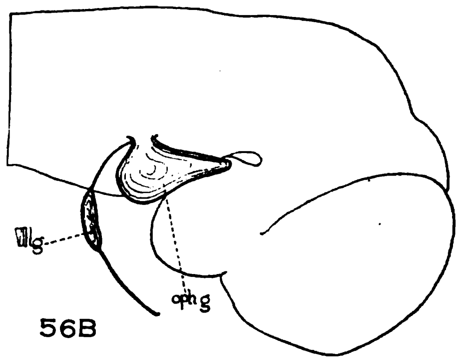

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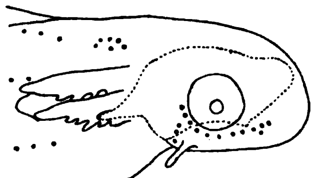
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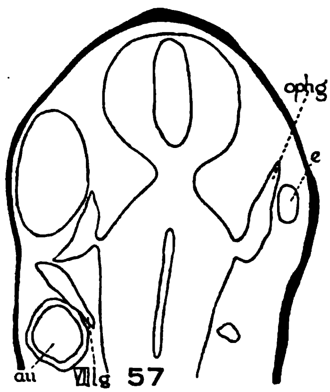

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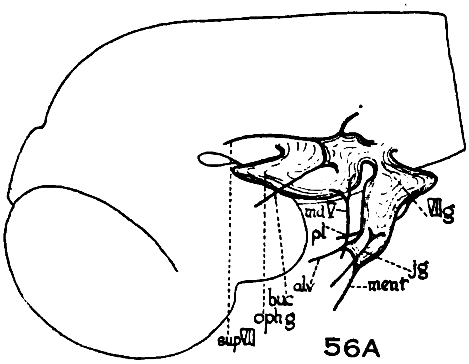

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Fig. 54 Camera-lucida drawing, showing by dotted line extirpated area of ectoderm one day after operation. The operation was done at stage 25 and included auditory and preauditory placodes as well as epibranchial placode of VII. × 10.

Fig. 55 Camera drawing of embryo shown in figure 54 twelve days later, showing extent of area of implanted blue ectoderm and absence of supra-orbital line. × 10

Figs. 56 a and b Lateral views of a reconstruction of the V and VII ganglionic complex of animal figured in 55, showing respectively, the normal and operated sides. The operated side shows a small VII lateral-line ganglion as only representative of VII. There is also no gasserian ganglion on operated side. × 50.

Fig. 57 Frontal section, showing only a small VII ganglion after removal at stage 26 of an area of ectoderm similar to that shown in figure 54. × 37.

is outlined from a case one day after operation (fig. 54). The operation was done at stage 25 and the extirpated area included the auditory placode as well as ectoderm anterior to it, although its anterior extremity did not include as much ectoderm as in the cases of the removal of the preauditory placode and supra-orbital primordium already described. The ventral extension of the extirpated area reached as far as the lower portion of the hyoid arch and in a few cases the mesoderm of the hyoid arch was exposed farther posteriorly than is the case in figure 54. The extension of the blue area at the time of killing, twelve days later, is shown in figure 55. All the groups of sensory lines are present except the supra-orbital group, although the sense organs which do appear on the right side of the head and lower jaw are not as prominent as on the normal side.

A reconstruction of the VII ganglionic components reveals only a small portion of this ganglion on the operated side (figs. 56 a and b). It lies lateral and slightly posterior to the ganglion of V and is elongated in the dorsoventral axis. As near as can be determined, a very slender root of fibers enters the brain near the posterior border of the V. The lower extremity of this small ganglion receives slender nerve fibers from the regions of lateral-line sense organs on the side of the head and the ventral side of the lower jaw. No palatinus, alveolaris, nor cutaneous portions of the jugularis nerves can be found on the operated side as are shown on the normal side (fig. 56 b). The ceratohyoid cartilage is slightly smaller on the operated side. The ganglionic portion of V in this individual seems to be concerned only with the ophthalmicus division. Another specimen which had a similar area removed during stage 25 was killed nineteen days after operation, and it also shows the same results.

A similar operation upon an embryo at stage 26–27 shows a small VII ganglion (fig. 57). The fibers from it seem to be lateral-line fibers to the sense organs on the lower part of the head. There is distinctly no palatinus VII, and whether the other components are entirely lacking or not cannot be exactly determined, for the animal was killed within a few days after the operation, and the small VII ganglion on the operated side has not developed

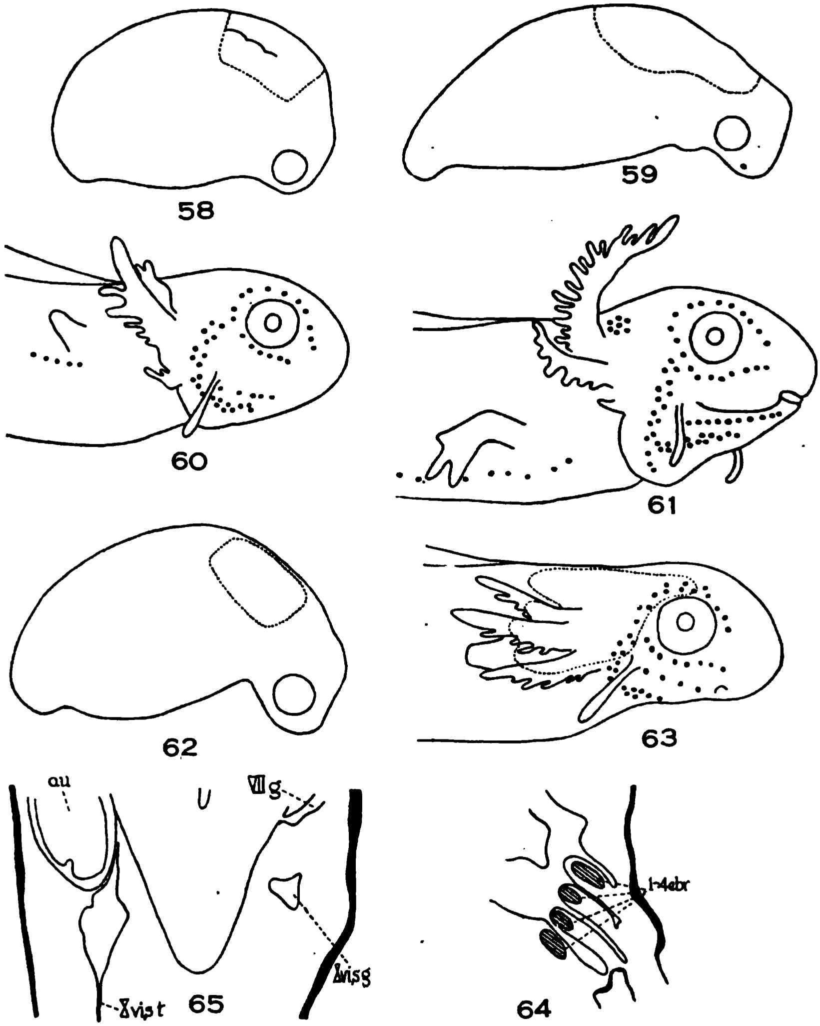


Fig. 58 Camera drawing, showing in dotted line and middorsal line in the area of ectoderm removed at stage 23 in excising postauditory lateral-line primordia and epibranchial placodes of IX and X. The auditory placode was also included. × 10.

Fig. 59 Camerad rawing, showing in dotted line the area of blue implanted ectoderm two days after operation, not including all the ectoderm along the posterior border of the gill swelling. × 10.

Fig. 60 Camera drawing ten days after operation, showing presence of a ventral body line of sense organs when posterior border of gill swelling is not removed as shown in figure 59. × 10.

sufficiently to say definitely how much is really lacking. The gasserian ganglion is much smaller in this individual. The ceratohyoid procartilage is normal on the operated side. In all cases where such an area as just described was removed there always appeared the same results, viz., a very small ganglion whose slender fibers supplied sense organs on the side of the head and lower jaw was the only representative of the VII ganglion.

6. Removal of epibranchial placodes of IX and X and postauditory lateral-line primordia. The operations were done around stages 21 and 26 and involved the removal of varying amounts of ectoderm posterior to, and often including, the auditory placode. The most satisfactory results were obtained in removing ectoderm outlined in the manner shown in figure 58.

Whenever the upper posterior border of the gill swelling was not removed (fig. 59) there appeared a small ventral body line of sense organs (fig. 60), although the dorsal and midbody lines were entirely absent. This occurred in many specimens and indicates, as shown in the descriptions of the normal material, that there is a separate placode for the ventral body line.

In a number of cases the occipital group of sense organs occurs (fig. 61), although the auditory vesicle was removed. The anterodorsal portion of the gill swelling was not well covered according to an observation of the blue ectoderm two days after the operation. The occipital or ventral body lines may persist after the operation, but only after operations done around stage 21. It appears that the area capable of regenerating these primordia is diffuse at this early stage.

Fig. 61 Camera drawing, showing only a few sense organs of occipital group when not all ectoderm is removed over anterodorsal portion of gill swelling. No dorsal nor midbody line of sense organs is present. × 10.

Fig. 62 Camera drawing, showing in dotted line twelve hours after operation at stage 26 the area of ectoderm removed, including all postauditory lateral-line primordia and epibranchial placodes. × 10.

Fig. 63 Camera drawing of embryo figured in 71, showing thirteen days after operation the extent of blue ectoderm and absence of postauditory lateral-line sense organs. × 10.

Fig. 64 Frontal section of the embryo shown in figure 63, showing normal branchial arches. × 37.

Fig. 65 Frontal section through similar level on either side, showing the small visceral ganglion of X on the operated side. × 37.

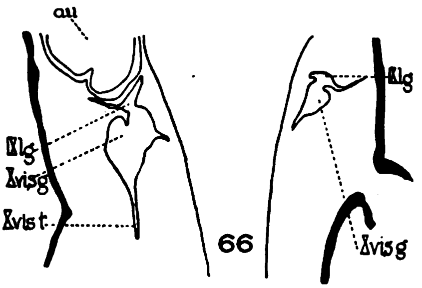
When the operation is done at about stage 26 and an area is removed as shown in figure 62, about twelve hours after operation, no postauditory primordia of the lateral-line sense organs appear. Only lateral-line sense organs anterior to the ear may be found. The extent of the blue area is marked out by dotted line (fig. 63).

An examination of certain typical specimens in this type of operation does not show such an extensive lack of ganglionic material as found in the regions of V and VII when all the crest cells are present.

The case illustrated in figure 63 shows normal branchial arches (fig. 64) and certain small ganglionic masses of IX and X. There is a very small X visceral ganglion (fig. 65). There are no first nor second branchial trunks. From the posteroventral portion of this ganglion a slender trunk comparable to the visceral trunk of the vagus passes posteroventrally alongside of a branchial blood vessel behind the anlage of the branchial bars to the posterior portion of the pharynx where its fibers are lost against the side of a blood vessel. There are no lateralis fibers in the visceral trunk because there are no lateral-line ganglia in the IX or X complex. A small visceral ganglion of IX lies below the level of X visceral ganglion at a lower level than on the normal side. Through it pass a few motor fibers which are given off to branchial muscles. A slender branchial trunk passes to the lateral side of the anlage of the levator arcus branchialis primus muscle to which it appears to give off a few motor fibers. Here it is lost in the first branchial arch and gives no evidence of containing sensory fibers. No special visceral fibers pass anteriorly toward the pharyngeal region.

Another specimen killed eighteen days after operation shows a few poorly developed sense organs in the occipital region which are supplied by a few nerve fibers from a very small IX lateral-line ganglion (fig. 66). A few lateralis fibers come from a small X lateral-line ganglion to innervate the posterior sense organs of the occipital group. The visceral ganglion of the vagus is somewhat smaller than on the normal side and gives off a visceral trunk which passes through a small ganglion on the median side of the fourth branchial arch. This ganglion seems to be a portion of

the vagus lateral-line ganglion which has possibly been formed by the ventral body-line primordium. The visceral trunk passes from this ganglion to the region of a small blood vessel, alongside of which it continues toward the posterior portion of the pharyngeal wall, where it is lost. Lateralis fibers also pass from this ganglion down to the ventral body line which is present for a short distance in this individual. Very slender first and second branchial trunks extend from the lower portion of the visceral



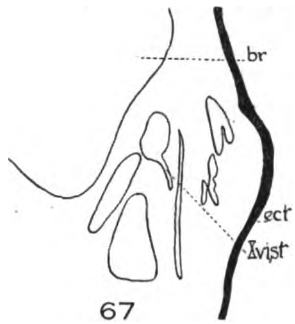


Fig. 66 Frontal section, showing the small visceral ganglion of X on operated side in front of which lies the IX lateral-line ganglion giving off fibers to occipital group of sense organs. A similar level on normal side shows a large IX and X ganglionic complex. The epibranchial placodes of IX and X were removed in this individual, but the occipital and ventral body-line primordia were not removed. × 37.

Fig. 67 Frontal section of a specimen from which epibranchial and lateral-line placodes of IX and X were removed, showing the X visceral ganglion and visceral trunk of X issuing from its posteroventral portion. It contains no lateralis fibers. × 37.

ganglion into the second and third branchial arches, but do not appear to carry any sensory fibers. The visceral ganglion of IX emerges close to the root of VII from a root which lies at a lower level than on the normal side. It gives off a trunk ventrally which passes into the first branchial arch running along close to a blood vessel for some distance until it is finally lost against the branchial muscles of this arch. This specimen also gives no evidence of containing visceral fibers in the sensory system.

Another specimen killed twenty-two days after operation shows all cartilages present, but slightly smaller in their dorsal portions while their lower portions are normal. Posterior to the ear there is but one occipital organ which is innervated by a single fiber coming from a small group of ganglionic cells—possibly the X ganglion. This ganglionic mass is very small and fused against a small vagus ganglion which is apparently only a visceral ganglion. Motor fibers may be seen issuing from the region of this ganglion to the branchial muscle. From the ventral side of this vagus ganglion a few short fibers extend to the region of blood vessels, where they are lost. Only the first branchial trunk of the vagus can be found and it contains only motor and general visceral fibers. The visceral trunk of the vagus ganglion may be seen issuing from the posteroventral portion of the ganglion (fig. 67). It carries no lateralis fibers, but is composed of general visceral fibers which are lost in the posterior region of the pharynx. The vagus lateral-line ganglion is entirely absent. No visceral ganglion of IX can be found.

These cases are typical of the findings from a study of a large number of individuals and the results seem to show that whenever the lateral-line placodes are removed no lateral-line ganglia are present, and when the ectoderm is removed from so large an area as to include the epibranchial placodes of IX and X and surrounding ectoderm no sensory fibers of the cutaneous and the special visceral system are present, while on the other hand the general visceral component is derived from the neural crest.

B. Removal of neural-crest cells

1. Contribution to mesodermal tissue. The neural crest was exposed to view by a longitudinal incision in the ectoderm beginning at a point below the middle of the third somite in the anterodorsal border of the pronephros and extending cephalad through the ectoderm of the dorsal third of the eye to the midline. The posterior extremity of the longitudinal incision was extended dorsally to the middorsal line, slanting slightly caudad. The ectoderm thus outlined was flexed dorsally hinging on the middorsal line.

An attempt to eliminate the neural-crest cells was made in several ways. By means of scissors and a fine spear-point needle the neural-crest cells were scraped away from the wall of the neural canal and, according to age, also carefully lifted off the mesoderm upon which they had migrated. The ectoderm was then turned back into its normal position, where it was weighted with glass rods and silver wires in the usual manner and allowed to heal. This treatment on one side only did not eliminate the possibility of the neural crest either from the sources of its origin on the operated side or from cells of similar groups wandering over from the unoperated side. Therefore, a series of embryos immediately after this operation was put into a cool chamber to retard development, and ten or twelve hours later the normal left side was similarly treated. In this type of double operation, however, the absence of normally developed crest cells makes a comparative study difficult.

A study of a large number of the first type of operation in which the crest cells were scraped away from one side only shows a large percentage in which regeneration of the crest cells had taken place, while many of the specimens which had undergone the double operation proved to be very oedematous in the gill region and unfit for study. Many of these individuals also showed complete regeneration of the neural crest.

In order to insure against the regeneration of the neural crest, a more extensive operation was performed. The ectoderm was flexed back in the manner already described and, after the neural crest had been carefully scraped away, the upper half of the neural tube on the right side was removed from the anterior border of the third somite to the anterior extremity of the brain above the eye. The number of cases available was considerably diminished by the high mortality attending the presence of such a large wound—only fifteen out of one hundred and fifty surviving. The high mortality was partially overcome by diminishing the area of operation by one-half. The ectoderm was flexed back upon the middorsal line so as to expose the crest cells over the branchial region. The crest cells along with the upper half of the neural tube from the anterior border of the third

somite to just in front of the ear were removed. In cases where it was advantageous the crest cells upon the mesoderm of the hyoid arch were included among the tissue that was removed. The number of recoveries from this kind of operation was far in excess of the number of deaths.

The neural crest over the mandibular and hyoid regions was removed along with the upper half of the neural tube from the posterior border of the ear to above the anterior border of the eye. The mortality in this type of operation far exceeded the recoveries. The ectoderm lying above the optic vesicles is very narrow, and when it is cut loose along its ventral border it soon shrinks toward the middorsal line, so that when the neural canal is removed the anterior extremity of the wound is often poorly covered. The healing is often incomplete at the anterior extremity of the head. The hole remaining, however small, is responsible for a large percentage of the deaths.

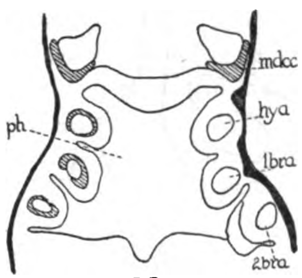
Among the group of embryos from which the neural crest has been removed by scraping there appear many cases which show varying degrees in the diminution of the size of the external gills. A very few showed slight deficiencies in the branchial cartilages.

The most favorable results were obtained from the specimens from which the neural tube was removed along with the neural crest. Among those cases in which this method was used to eliminate the crest cells over the branchial region and occasionally over the hyoid region there are recorded a number of specimens killed at various stages in development after the operation.

Case 1. The operation was done at about stage 26 and the embryo was killed three days later. It had not developed very rapidly as it remained in the cool chamber up to the time of killing. Frontal sections through the mesoderm of the arches on the normal side show that the crest cells are completely surrounding them. A section at the level of the middle of the optic cups (fig. 68) shows a complete absence of crest cells on the mesoderm of the second branchial arch on the operated side. On the median side of the mesoderm of the first branchial arch only a few crest cells appear, which are continued in a few sections fur-

ther ventrally and then cease. They may be remnants of crest cells which adhered to the outer border of the mesoderm at the time of operation. However, their number is insignificant compared to the amount of similar cells on the normal side. The crest cells around the mesoderm of the hyoid arch are also considerably less in amount than on the normal side.

Case 2. The operation was done at the same stage as in the previous case and the specimen was killed after six days. A frontal section (fig. 69), although somewhat oblique, shows in the



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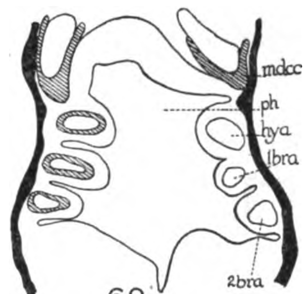

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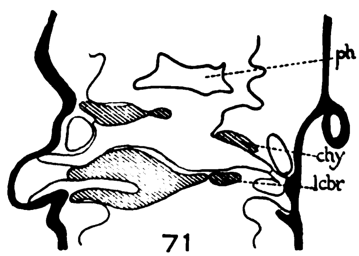

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Fig. 68 Frontal section through the mesoderm of hyoid, first and second branchial arches, showing, three days after removal of crest cells, no crest cells on right side. × 37.

Fig. 69 Frontal section of specimen killed six days after removal of crest cells in hyoid and branchial regions, showing a distinct lack of crest cells on right side around the mesoderm of hyoid and first and second branchial arches. × 37.

Fig. 70 Frontal section of specimen killed nine days after removal of crest cells over the hyoid and branchial regions, showing only three procartilage cells in the third branchial arch. × 37.

Fig. 71 Frontal section at a lower level in the same specimen as in figure 70, showing only a few procartilage cells in the hyoid and first branchial regions. × 37.

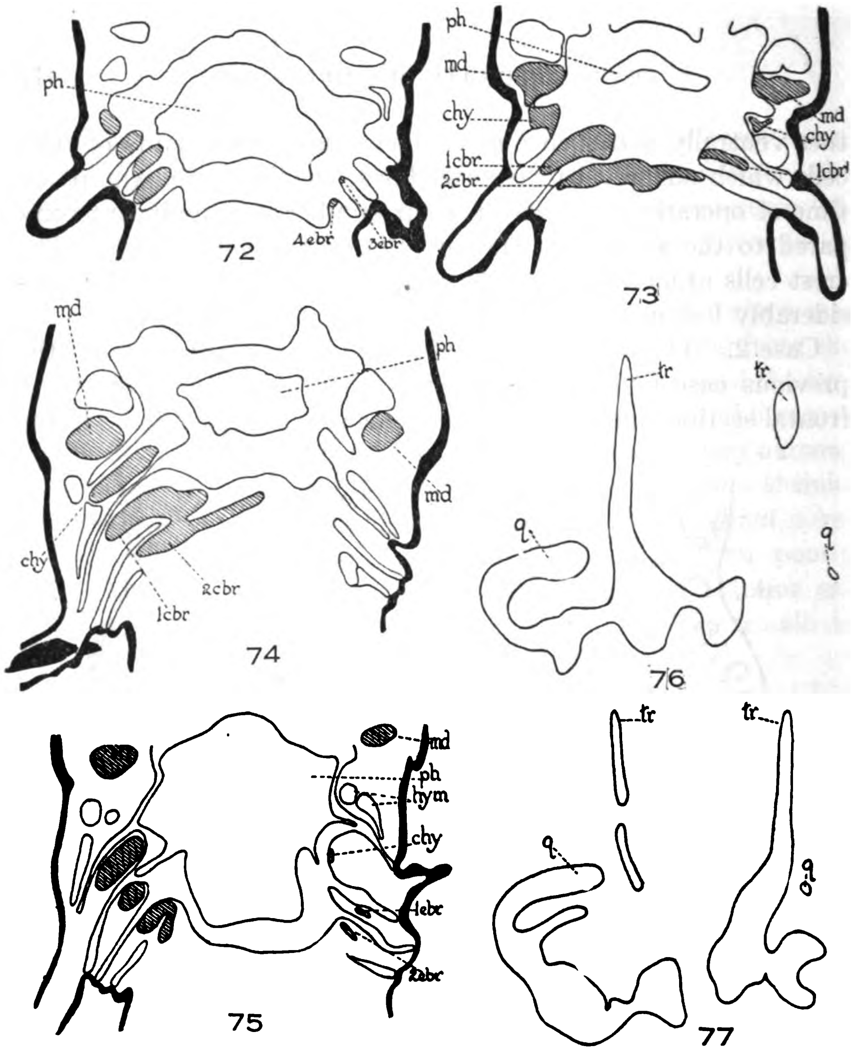


Fig. 72 Frontal section through procartilages of the arches of a specimen killed fourteen days after operation, showing only a few procartilage cells in the third and fourth branchial arches. × 37.

Fig. 73 Frontal secton at a lower level in same specimen as in figure 72, showing only a few procartilage cells in the hyoid arch and a small number in first branchial arch on operated side. × 37.

Fig. 74 Frontal section of specimen killed seventeen days after removal of crest cells from hyoid and branchial regions, showing absence of ceratohyal and first and second ceratobranchial cartilages on the operated side. × 37.

Fig. 75 Frontal section of a specimen killed eleven days after operation, in which an attempt was made to remove all the crest cells,.showing only rudiments of cartilages in the hyoid, first and second branchial arches. The quadrate is smaller than normal. × 37.

Figs. 76 and 77 Frontal sections at two levels in the same specimen, showing the small size of the right quadrate cartilage. × 37.

region of the mesoderm of the hyoid and the first and second branchial arches a distinct lack of crest cells on the operated side, while on the normal side they are abundant and still surround the mesoderm of the arches.

Case 3. A similar specimen was killed nine days after operation, when it had reached about stage 37. The external gills at this stage have become prominent finger-like processes, but have not developed branches. A frontal section of this specimen (fig. 70), although obliquely cut, shows to even more advantage the comparison of the two sides, for the level of the operated side is lower where there would be normally an abundance of procartilage-forming cells. There are but three crest cells in the region of the third branchial arch, while the others lack cells of this kind altogether. This is the condition in sections above this level. The crest cells in the hyoid region are also noticeably less in amount than on the left side. At a lower level (fig. 71) the difference is more striking, for the hyoid, the first, and part of the second branchial regions have very few cartilage-forming cells compared with the abundance on the normal side. Loose connective tissue in the external gills on the operated side is also subnormal in amount.

Case 4. This specimen was killed fourteen days after the operation, when it had reached about stage 39. The gills on the operated side are smaller than on the normal side and their connective-tissue cells are less numerous. Compared with the normal side, there are only a few procartilage cells in the third and fourth branchial arches (fig. 72). When followed farther ventrally (fig. 73), the few loose cells on the right side increase but slightly in number and always remain much more loose in arrangement as they approach in the midline the normal compact procartilage. The procartilage in the hyoid region is also much less in amount.

Case 5. This specimen was killed seventeen days after operation and also shows a decided diminution in the size of the gills on the operated side. A frontal section shows well-formed branchial cartilages on the left, while on the right a few cells form a small cartilage only in the third gill arch. When followed

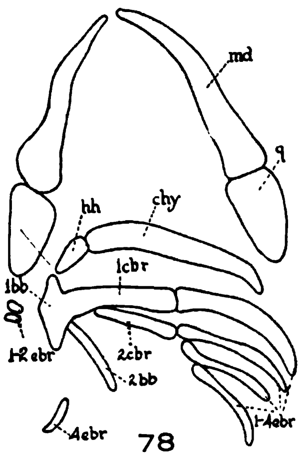
farther ventrally, very small rudiments of the second, third, and fourth branchial cartilages appear, which, after they approach each other at their ventral extremities, become completely lost and do not approach in the midline the cartilages of the normal side. The hyoid cartilage is completely absent. The first and second ceratobranchials and the ceratohyoid are absent on the operated side (fig. 74). The first and second basibranchials are present.

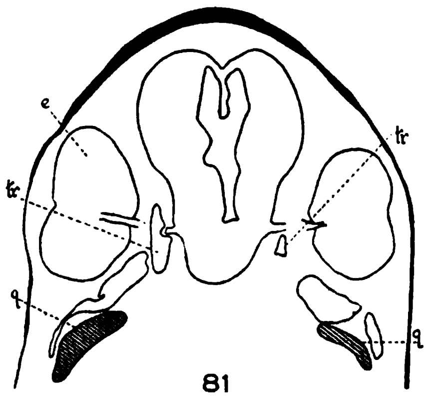
A specimen in which all the crest cells were included in the operation along with the upper half of the neural tube was killed eleven days after operation, and it shows many deficiencies in the visceral skeleton. Very small first, second, and third epibranchial cartilages are found on the operated side (fig. 75); they soon disappear as the sections are followed ventrally. The quadrate in this and other sections is smaller than on the normal side. The hyohyal, ceratohyal, and all the ceratobranchial cartilages are entirely absent. The mandible is smaller on the right side. The first basibranchial cartilage is present but small and lies toward the right of the midline, while the anlage of the second basibranchial is apparently normal. No change can be seen in the anterior portions of the trabeculae.

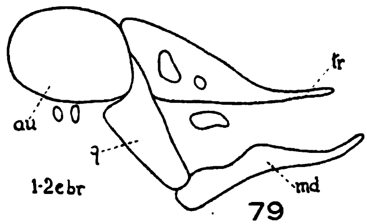
A similar attempt was made in another specimen, but due to incomplete removal of crest cells in the branchial region regeneration had taken place. However, seventeen days after operation it showed externally a diminution in size of the right side of the lower jaw. A section at a level with the lower border of the ear and the optic nerve shows a remnant of the quadrate very small (fig. 76) when compared with figure 77, a similar level on the left side. At the level of the lower border of the right eye the quadrate cartilage appears again for a short distance and extends toward the articular end of the mandible. As the sections are followed farther ventrally, the mandibular cartilage on the right side is seen to be smaller than on the normal side.

Another specimen was operated upon at stage 26 and killed twenty-one days later. At this stage it is impossible to remove successfully all of the crest cells which have migrated down over the mesoderm of the mandibular arch. The external gills and

lower jaw on the operated side showed an abnormal development. A dissection of this individual was made after the cartilages had been stained with methylin blue and shows (fig. 78) only three small branchial cartilages on the right side while the first and second basibranchial cartilages are present, but abnormally placed to the right of the midline. The quadrate and mandibular cartilages are somewhat smaller (figs. 79 and 80). There is not much difference in the size of the anterior portions of the trabeculae.







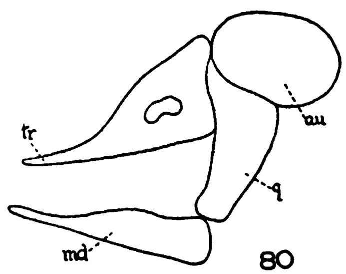


Fig. 78 Showing a camera-lucida drawing of a dissection of cartilages stained in methylene blue. Killed twenty-one days after operation. An attempt was made to remove all the neural crest in this specimen. Only three small branchial cartilages appear on the operated side. The first basibranchium is less developed on the right side, while the second basibranchium is a slender rod and displaced. The right mandibular cartilage also appears smaller than the normal. × 23.

Figs. 79 and 80 Camera drawings of lateral views of a dissection of specimen figured in 78, showing, respectively, operated and normal sides. The quadrate and mandibular cartilages are smaller on the operated side. × 23.

Fig. 81 Frontal section, showing in another specimen at the level of the optic nerves relative sizes of the quadrates when the crest cells have been removed at stage 24. × 37.

Another individual was operated on at stage 24 and the crest cells along with the upper half of the neural canal were removed from the hyoid and mandibular regions. There was evidently a regeneration of the crest cells, but the specimen showed certain deficiencies in the visceral skeleton. At a level with the optic nerves a section (fig. 81) shows a small quadrate cartilage on the operated side. In levels above and below this section the difference in the size of the two cartilages is even more striking. The anterior portions of the trabeculae show a difference in size. The anterior portion of the trabecula on the normal side extends somewhat beyond the level of the optic nerve, while on the operated side it extends only to the posterior border of the optic nerve. The ceratohyoid cartilage is poorly developed and joins with the first basibranchial posterior to its normal position. The mandibular cartilage in this individual also shows a deficiency in size.

It was found in the large number of operations made in removing the neural crest that the most favorable stage was around stage 26. However, this applies only to the branchial and hyoid regions. Here their removal is a very simple matter and no damage to the mesoderm need be expected, for the crest cells lie loosely upon the mesoderm and do not extend very far ventrally. In the mandibular region the elimination of crest cells is more difficult, for several factors are involved. From stage 23 to 26, when the regeneration of the neural crest is most persistent, a very rapid ventral growth takes place far in advance of the ventral proliferation of crest-cell groups in the hyoid and branchial regions. The crest cells lie tightly against the mesoderm of the mandibular arch, and in order to remove these cells considerable damage is caused to the mesoderm. The wound is very extensive ventrally, and the pulling of the cut edge of the ectoderm near the optic vesicle, accompanied by the large cavity made in the anterior portion of the brain, always resulted in the death of such individuals. It is hoped that by doing a large number of operations in this region a few specimens may survive which will show a complete removal of the crest cells over the trigeminal region.

An examination of the specimens done at the earlier stage shows a deficiency of the mandibular and quadrate cartilages, but not a complete absence. One case shows a deficiency in the anterior portion of the trabecula. In the branchial and hyoid regions it is quite evident that the branchial cartilages, the ceratohyal, the hyohyal, and the first basibranchial are derived from the wandering neural crest.

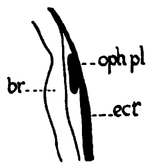
2. Contributions to ganglionic components. a. Contribution to V and VII. As already shown in the cases described, there is no condition in which the crest cells in the region of the trigeminus were entirely removed, although in the other regions they have been almost entirely eliminated.

A specimen operated upon at stage 25 was killed two days after operation and shows on the operated side a portion of the ophthalmic placode which is giving off cells, even though few crest cells may be found in this region. It lies close to the brain (fig. 82) while farther ventrally placodal cells from the posterior portion of the VII lateral-line placode are projecting medially to form the lateral-line ganglion (fig. 83). At its anterior portion it is contiguous with a small group of cells which belong to the gasserian placode. The epibranchial placode of VII at this stage is prominent, but has split off no cells on either side.

Although the crest cells have regenerated to a small degree in the trigeminal regions of two other specimens killed within a few days after operation, placodal cells may be seen contributing in large numbers to an ophthalmic ganglion. The condition of the ganglion is practically the same as on the normal side. One of the specimens shows a large well-formed ophthalmic ganglion (fig. 84), but the number of crest cells on the mesoderm of the mandibular arch farther ventrally is far less than on the normal side. Although there are but few crest cells on the mesoderm of the hyoid arch of this specimen, the VII lateral-line ganglion is present and normal (fig. 84). This level does not show the comparative size of the two ganglia. The visceral portion of the VII seems to be a little smaller than on the normal side.

Another typical case killed eleven days after operation shows deficiencies in the mandibular group of crest cells and an entire

absence of the ceratohyal cartilage. The ophthalmic ganglion is large and normal in size, although it is situated somewhat dorsal to its normal position (fig. 85). The portion of the lateral-line ganglion of VII which supplies the supra-orbital and infra-orbital group of sense organs also appears in this level and is normal in size as well as the rest of the lateral-line ganglion.



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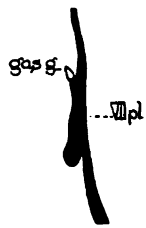

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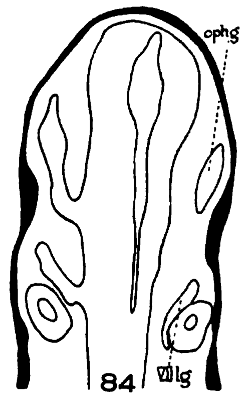

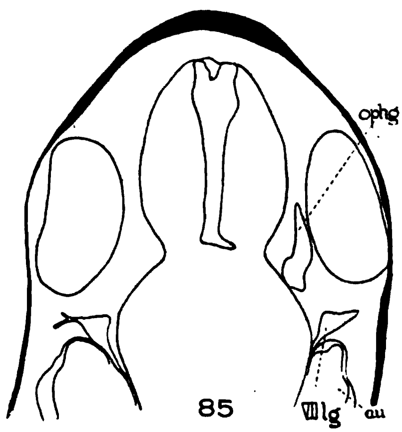

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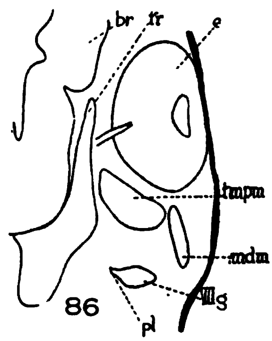

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Fig. 82 Frontal section, showing the ophthalmic placode two days after the crest cells have been removed. × 37.

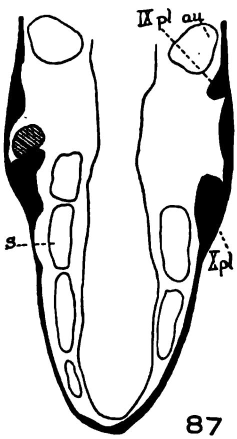
Fig. 83 Showing in the same specimen the posterior portion of VII lateral-line placode giving off placodal cells. At its anterior extremity is shown the contact of the gasserian ganglion with the ectoderm. × 37.

Fig. 84 Showing a normal VII lateral-line ganglion when crest cells have been removed from the hyoid arch. × 37.

Fig. 85 Frontal section, showing a large ophthalmic ganglion after crest cells had been removed at an early stage. × 37.

Fig. 86 Frontal section further ventrally in the same individual, showing the palatinus VII at the point of leaving the ganglion. × 37.

The gasserian ganglion is slightly smaller than on the normal side and lies somewhat separated from the ophthalmic ganglion. The visceral portion of VII is a little smaller and no definite alveolar nerve can be found. The special visceral or palatinus VII is present (fig. 86).



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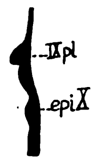

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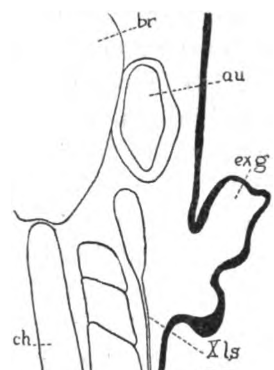

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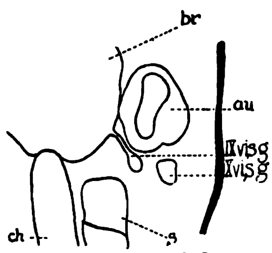

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Fig. 87 Frontal section of a specimen killed three days after removal of crest cells over the branchial region, showing lateral-line placodes of IX and X forming ganglia. Between them in ectoderm lies the epibranchial placode of X. On the normal side lies a group of crest cells near the epibranchial placode of X, possibly forming a portion of visceral ganglion of X. × 37.

Fig. 88 Portion of a frontal section in a specimen killed a few days after removal of crest cells, showing normal epibranchial placodes of IX and X. × 37.

Fig. 89 Showing a large vagus lateral-line ganglion and ramus lateralis superior vagi in a specimen killed eleven days after an attempt had been made to remove all crest cells on right side. × 37.

Fig. 90 Showing in the same specimen the IX visceral ganglion. × 37.

b. Contribution to IX and X. A specimen killed three days after operation shows on the operated side a large lateral-line ganglion placode of IX just anterior to the epibranchial placode of X (fig. 87). On the anterior surface of the large vagus lateral-line placode are a number of loose placodal cells given off from the placode. No crest cells are found in the branchial region of the operated side. On the normal side anterior to the vagus

placode is a mass of crest cells near the ectoderm and epibranchial placode of X. These are crest cells which possibly give rise to a portion of the visceral ganglion, for when followed ventrally they lie close to the epibranchial placode of X.

A specimen killed five days after operation shows on the operated side a large normal lateral-line ganglion. The placode of the lateral-line ganglion of IX seems to be a little less advanced in development than on the normal side. Farther ventrally (fig. 88) loose masses of cells are being given off from the epibranchial placodes of IX and X and are not of crest-cell origin, for there are no crest cells in the branchial region of the operated side.

A specimen killed eight days after operation shows a large vagus lateral-line ganglion, in front of which is a portion of a small visceral ganglion. The ganglia are still made up of loose cells and nerve fibers from the visceral ganglia cannot be determined. Posterior to the auditory vesicle on the operated side is a small lateral-line ganglion which lies above the small visceral ganglion of IX which is connected with the epibranchial placode. Only a very few scattered crest cells appear on the median surface of the mesoderm of the branchial arches. A number of other specimens killed between eight and ten days after operation show small visceral ganglia, which are derived from the epibranchial placodes, and perfectly normal IX and X lateral-line ganglia. In these cases also there are only a few loose crest cells in the branchial region on the operated side.

A typical specimen killed eleven days after operation shows a large vagus lateral-line ganglion with the ramus lateralis superior vagi nerve extending posteriorly to innervate the body line of sense organs (fig. 89). The anterior portion of this ganglion is part of the visceral ganglion of X. From it pass slender fibers to the epithelium of the second external gill and also a number of motor fibers to the branchial trunk of the vagus can be seen in the second branchial arch. It gives off motor fibers to the branchial muscles, and when followed ventrally into the branchial arch it is lost near the epithelium on the pharyngeal side. There is no definite second branchial trunk. As the superior lateralis

vagi nerve leaves the ganglion, fibers are continued ventrally, as on the normal side, to form the visceral trunk of the vagus. A few motor fibers are given off along its path, and when followed farther ventrally it is finally lost near the wall of the pharynx. No ramus intestinalis from the visceral trunk can be determined. In the dorsal portion behind the ear a number of cutaneous fibers may be seen, along with the lateralis fibers, to innervate the skin. The visceral portion of IX may be seen at the lower border of the ear coming off from the root of IX which is at a lower level than on the normal side (fig. 90). The visceral ganglion is small and gives off no trunk to the first branchial arch, but ventral to the ear capsule it sends out a nerve which passes some distance along the median border of the internal ceratohyoid muscle, where it is followed ventrally until its fibers are lost against the wall of the pharynx. The only visceral fibers that can be identified on the operated side appear to be of the special visceral system. The other fibers which appear in the IX and X ganglionic complex on the operated side are of the lateralis and general cutaneous systems. In the ventral positions of the third and fourth branchial arches only very small rudiments of cartilages appear, which shows that very few crest cells remained which could have contributed to the visceral ganglia.

It appears from the study of the specimens described that the only contribution of the crest cells to the ganglionic complex of IX and X is to the general visceral component.

DISCUSSION

It has been shown in the study of early stages of Amblystoma embryos that extensive contributions from the lateral ectoderm take part in the formation of cranial ganglia, and the experimental analysis of the problem has shown how small a part the crest cells play in the formation of these ganglia. The facts which the experiments present lead to the conclusion that the general cutaneous system is derived entirely from placodes. In the trigeminal region there are two definite placodes concerned with the formation of the V; that in the case of the ophthalmic division is the larger, while that of the gasserian is the smaller and

of shorter duration and, therefore, difficult to follow. The earliest stage in which Coghill ('16) described the early contact of the ophthalmic ganglion with the ectoderm corresponds to about stage 34, i.e., a stage between those shown in figures 6 and 7. At the point of contact lies the placode which can be last seen at this stage. The actual contribution of placodal cells to the ganglion must be observed in earlier stages than this, at a time when crest cells are still very numerous in this region. This condition has been the factor which has caused investigators to overlook the placodal contribution. Coghill's observation that during this contact with the ectoderm the ganglion makes its connection with the brain adds further morphological evidence that the ganglion is of placodal origin. Judging from the many similarities in Amblystoma and Necturus, it is obvious that Platt was correct in assuming an ectodermal contribution to the ophthalmic ganglion, although she has confused the placodal and crest cells in this region and incorrectly interpreted part of these placodal cells as contributing to the 'mesectoderm.' The ectodermal cells which Goette ('14) describes being given off above the optic vesicles in Siredon (Amblystoma tigrinum) and Torpedo are likewise cells of the ophthalmic placode and not contributions to the wandering 'ectomesoderm.' It has been shown that when the ophthalmic placode is entirely removed in early stages of Amblystoma there is a complete absence of the ophthalmic ganglion and the ophthalmicus profundus V nerve (figs. 40 b and 43). Such cases always show but little disturbance of the crest cells in the trigeminal region. In control operations in which the ectoderm was removed in the usual manner and then replaced there appeared normal ophthalmic ganglia and nerves, which shows that the placode is necessary for the formation of the ganglion. When a small portion of the ectoderm over the eye is left a very small displaced ganglion is often found (fig. 45 b). In this respect it is similar to other partially removed placodes. On the other hand, when the ophthalmic placode is left intact and the crest cells have been disturbed as much as possible by an attempt to remove them, an apparently normal ganglion is present, and although there has been a regeneration of the crest cells

it shows that an extensive disturbance of the crest cells as early as stages 21 and 23 does not inhibit the growth of the ganglion.

As Coghill ('16) has already shown in Amblystoma, there is an early contact of the gasserian ganglion with the ectoderm. At the point of contact near the anterior border of the preauditory placode (figs. 21 and 26) is a small thickening in the ectoderm which can be followed only through stages 28 to 30. Although this condition has not been described in any other forms, it is quite possible that it does exist, but has been overlooked on account of its small size and short duration in a region where the crest cells are very abundant. Among the cases where the removal of the preauditory placode included ectoderm near the posterodorsal border of the optic vesicle there occurred two cases (fig. 56 b) in which there was no gasserian ganglion. When smaller areas of ectoderm were removed from the posterodorsal region of the eye there often occurred small gasserian ganglia. This was possibly due to the fact that not all of the placode had been removed. In one case where there was a deficiency in the crest cells on the mandibular arch after the crest cells had been removed a smaller gasserian ganglion was observed. In this case it seems quite possible in the light of the control and other operations that the gasserian placode was injured when the ectoderm was reflected at the time of operation.

The remaining portion of the general cutaneous system of the cranial nerves is to be found in the X. In the observations reported in this paper no definite distinction could be made in the early stages between the small general cutaneous and the visceral ganglia of X. Coghill ('16) has observed that during its early contact with the ectoderm, the cutaneous ganglion of X has no connection with the brain. However, when a large area of ectoderm was removed containing the epibranchial placodal regions of IX and X no definite general cutaneous fibers could be found. This leads one to conclude that the general cutaneous portion of the vagus complex is derived from the lateral ectoderm and the early contact of the small general cutaneous ganglion of X, which was described by Coghill, is the indication of a placode in the ectoderm which gives rise to that ganglion. This fact falls in

line with the results obtained in the removal of placodes in the trigeminal region and shows that the general cutaneous system is placodal in nature and not, as Landacre ('10) has suggested, of neural-crest origin.

From the results obtained in the series of experiments recorded in this paper there can be no doubt that the lateral-line sensory system in Amblystoma is derived entirely from placodes. The study of the preauditory placode shows that a large part of the lateral-line ganglion of VII is formed from this placode and from its anterior end arises the supra-orbital primordium of sense organs. The other lateral-line primordia in the head region are separate in origin as in Necturus and also contribute to the VII lateral-line ganglion. At no time is there any condition such as that described in Lepidosteus by Landacre and Conger ('13) in which the preauditory placode begins to disintegrate at the time when the first trace of the lateral-line primordium can be detected. It is quite possible that Landacre and Conger were misled in this interpretation of the preauditory placode, for, according to their description, it apparently arises very early, and although they describe no cells being given off from the placode, it seems probable that there may have been an early contribution which was unobserved.

In the case of the postauditory lateral-line primordia, the study of experimental as well as of normal material shows that the three trunk lines of sense organs have separate primordia, and in this respect Amblystoma is similar to Necturus. The experimental results show that the occipital group of sense organs appeared in a few cases where the ectoderm in the anterodorsal portion of the gill swelling (fig. 61) was not entirely removed, although the ear was entirely removed. This condition implies the independence of the occipital primordia from the auditory placode and also indicates in embryos close to stage 21 the ability of the ectoderm in the anterodorsal region of the gill swelling to give rise to occipital primordia. The complete removal of the postauditory lateral-line primordia was not only accompanied by the absence of the groups of sense organs, but by an entire absence of the lateral-line ganglia. When only a few sense

organs appeared, correspondingly small lateral-line ganglia were present which innervated those sense organs. No evidence can be obtained that crest cells contribute to the formation of lateral-line ganglia. The morphological studies are misleading in this respect, for in many cases the close arrangement and contact of the early crest cells and placodes make an interpretation of the exact contribution of the two kinds of cells difficult to understand.

In the study of the normal embryos the epibranchial placodes of VII, IX, and X could be located as early as stage 26–27 (fig. 4) and their contributions to ganglia could be followed up to stage 36. The observations of these placodes agree in many respects with Coghill as to the placodal relation and contribution to the visceral ganglia. The removal of epibranchial placodes was found to be accompanied by a distinct lack of gustatory fibers in VII, IX, and X with no apparent disturbance to the general visceral system. In this respect the experimental results agree with Landacre's explanation of the function of the epibranchial placodes in Lepidosteus, viz., that they give rise to special visceral ganglia of VII, IX, and X.

Goette ('14) expresses the belief that the epibranchial and lateral-line placodes form, with the crest cells, a syncytial mass of cells out of which ganglia and nerves are formed which later join themselves up with the brain. Studies of experimental and normal amblystoma embryos show that certain definite portions derived from placodes and crest cells, although they mingle with each other, maintain their identity and are not to be considered a syncytial mass at any time.

It has also been shown in Amblystoma that the neural crest originates from the dorsal portions of the contiguous surfaces of the neural folds at the time of the closure (fig. 13). These crest cells were followed by means of their difference in pigment and by the presence of fine yolk granules in their cytoplasm as they migrate ventrally over the mesoderm of the arches, always remaining separate from the ectoderm. The wandering mass of ectoderm is of crest-cell origin only and does not in Amblystoma receive any contribution from the ectoderm on the lateral surfaces of the head. Platt's descriptions of Necturus show clearly

that the positions of the proliferating lateral ectoderm correspond to placodal regions and since the migration of crest cells soon produces a scarcity of these cells in the dorsal region of the neural canal and an abundance of crest cells in the region where placodal cells are given off, a condition is brought about which would lead to a confusion as to the origin of the wandering 'mesectoderm.' Aside from the different interpretation in the origin of the 'mesectoderm' in Amblystoma the manner of the formation of the branchial cartilages with the exception of the second basibranchial agrees with the description which Platt ('97) gives of the branchial cartilages in Necturus. This is fully in accord with Landacre ('21). The branchial cartilages with the exception of the second basibranchial have been conclusively shown to have their origin in the neural crest. At the time when this skeleton begins to take on a cartilaginous appearance the first basibranchial extends a short distance posteriorly from the attachment of the ceratobranchial cartilages. This condition is somewhat misleading for it gives the appearance that the second basibranchial is a posterior outgrowth from the first basibranchial. However, such is not the case, for a study of embryos about stage 42 conclusively shows that the second basibranchial is formed out of mesoderm near the anterior wall of the pericardial chamber and that this cartilage retains large mesodermal yolk granules for a long time after the branchial skeleton from the neural crest has lost all of its yolk granules (fig. 37). Amblystoma in this respect agrees with Landacre's ('21) description of Plethodon. The experimental results show that some of the neural crest is incorporated in the connective tissue of the external gills as well as in the balancer, as Harrison ('21) has shown. How much more of the connective tissue in the branchial region is formed from the crest cells is impossible to determine at this time.

In the case of the mandibular and quadrate cartilages the experimental results, as already stated, did not show as conclusively as the findings from the study of normal embryos that they are derived from the neural crest, because of the difficulty in eliminating the crest cells from the trigeminal region. Nevertheless, the results do show that there was a decided diminution

in the size of those cartilages when the crest cells were removed. Although it was difficult to determine from the studies of normal embryos what became of the neural crest migrating over the dorsal and anterior margins of the optic vesicles, one case (fig. 81) in which the crest cells were removed in the trigeminal region seems to show conclusively that they form the anterior portions of the trabeculae. This is in accordance with the findings of Platt ('97) and Landacre ('21).

In no case do the crest cells enter into the formation of any part of the branchial musculature as described by Goronowitsch ('93). The musculature of the visceral arches is formed entirely from the mesoderm of those arches. No portion of the skull other than the anterior portion of the trabeculae is formed from the neural crest.

The only contribution of the neural crest to the formation of cranial ganglia is probably to the general visceral portions of VII, IX, and X. This conclusion is substantiated by the fact that when the crest cells are removed from the branchial and hyoid regions, there is a distinct lack of general visceral fibers, while the gustatory, lateralis, and general cutaneous fibers are normal.

SUMMARY

1. Above the optic vesicle in early stages of Amblystoma there is an elongated ophthalmic placode which gives off cells to the formation of the ophthalmic ganglion. When the ectoderm including this placode is removed as early as stage 23, the ophthalmicus profundus V nerve and ganglion are absent.

2. Near the anteroventral border of the supra-orbital lateral-line primordium is a small gasserian placode of brief duration which can be followed through stages 28 and 30. When a large area of ectoderm is removed from the region posterodorsal to the eye, deficiencies of the gasserian ganglion are produced.

3. Lying close to the anterior border of the auditory placode is a prominent placode elongated in a direction toward the dorsal extremity of the hyomandibular cleft. It can be located as early as stage 25. When ectoderm in this region is removed, even

before the appearance of the placode, the supra-orbital line of sense organs is absent as well as a large part of the VII lateral-line ganglion. The small remaining VII lateral-line ganglion in such cases gives rise to lateralis fibers, which innervate the group of sense organs on the lower jaw, and also slender fibers to the infra-orbital group.

4. The supra-orbital primordium of lateral-line sense organs arises from the anterior extremity of the VII lateral-line ganglion placode.

5. The supra- and infra-orbital and hyomandibular primordia of lateral-line sense organs have separate seats of origin.

6. The ventral hyomandibular and mandibular groups of lateral-line sense organs also appear to have separate seats of origin.

7. The epibranchial placodes of VII, IX, and X give off cells which become incorporated in the visceral ganglia, and when these placodes are removed from the ectoderm in early stages (23–26) no special visceral ganglia nor gustatory fibers can be found.

8. The complete removal of ectoderm in the region of IX and X which includes all the primordia of the lateral-line system is accompanied by a complete absence of lateral-line ganglia. When only partially removed, small lateral-line ganglia are produced.

9. Large areas of ectoderm removed from the region of IX and X also show an absence of cutaneous fibers as well as visceral sensory fibers.

10. The lateralis and special visceral ganglia are derived entirely from placodes, and the general cutaneous, for the most part if not entirely, is also derived from placodes.

11. The neural crest cells arise from the dorsal portion of the neural tube at the points of the fusion of the neural folds. They can be distinguished as early as the closure of the folds, and from this region they can be followed by their difference in pigmentation from the surrounding tissue and by the presence of small yolk granules in their cytoplasm as they descend upon the mesoderm of the visceral arches around which they wrap themselves and

later become arranged on the median surfaces of the arches where they form cartilaginous tissue.

12. The wandering mass of 'mesectoderm' is of neural-crest origin and in no region is it augnented by a contribution from cells of the lateral ectoderm.

13. The removal of neural crest in the branchial and hyoid regions is accompanied by smaller external gills and marked deficiencies in the branchial and hyoid cartilages. The hyohyals, ceratohyals, ceratobranchials, epibranchials, and first basibranchial are formed from the wandering neural crest, while the second basibranchial is formed from mesoderm near the anterior wall of the pericardial chamber. In the ganglionic regions the neural crest appears to form only the general visceral components.

14. The removal of neural crest in the trigeminal region shows no complete absence, but deficiencies in the size of the quadrate and mandibular cartilages. However, there is no doubt that these cartilages are formed from crest cells. The regeneration of the crest cells always occurred in the operation of these specimens because they were necessarily confined to very early stages.

15. The neural crest in the trigeminal region which migrates over the anterior border of the optic vesicles apparently gives rise to the anterior portion of the trabeculae.

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SUBJECT AND AUTHOR INDEX